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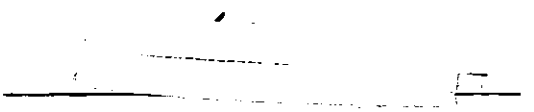

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THE ISOLATION AND IDENTIFICATION OF ORGANIC COMPOUNDS FROM RHUS GLABRA

A THESIS

Presented to

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by

Robert Arthur Heckman

In Partial Fulfillment

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THE ISOLATION AND IDENTIFICATION OF ORGANIC COMPOUNDS FROM *RHUS GLABRA*

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GLOSSARY OF ABBREVIATIONS

d	Chromatography column inner diameter.
EGA	Ethylene glycol adipate, stationary phase.
EGS	Ethylene glycol succinate, stationary phase.
EGSS-X	Methyl silicone - EGS copolymer, stationary phase.
GLC	Gas-liquid chromatography.
h	Chromatography column height.
p_i	Column inlet pressure.
SE-30	Methyl silicone, stationary phase.
t	Column temperature.
TLC	Thin-layer chromatography.
t_R	Retention time for solute in minutes measured from the solvent peak.

SUMMARY

The results of biological screening of ethanol extracts of the fruit of Rhus glabra (smooth sumac) that were prepared in this laboratory indicated that the fruit contained some biologically active principles. Attempts to isolate basic fractions from the ethanol and acidified ethanol extracts of the fruit failed; these results precluded the presence of alkaloids. These extracts were also devoid of steroidal saponins (they possessed no hemolytic activity). Positive identifications of potassium hydrogen L-malate, gallic acid, and pyrogallol were made from the ethanol extracts. These compounds had been isolated by earlier workers from the fruit of Rhus glabra and Rhus typhina.

Successive extraction of whole Rhus glabra fruit with petroleum ether and with chloroform yielded extracts that represented 4.5 and 2.3 per cent, respectively, of the partially dried fruit. The nonsaponifiable portions of these extracts were small (5-7%) and ca. one-third of the nonsaponifiable material derived from the petroleum ether extractives consisted of a mixture of n-alkanes. Repetitive chromatography of the crude n-alkanes produced a white crystalline mixture which melted over a relatively narrow range. GLC analyses of this material indicated the presence of all the homologues from C_{15} to C_{33}; the most abundant homologue was n-nonacosane. The mixture was largely comprised (ca. 96%) of n-alkanes having an odd number of carbon atoms. The nonsaponifiable portion of the chloroform extractives was not investigated.

GLC analysis of the methyl esters derived from the fatty acid

components (both free and combined) of the above extracts indicated that palmitic acid was present in largest amount. Palmitic and oleic acids together comprised 96.2 per cent of the mixture of fatty acids derived from the chloroform extractives. These mixtures also contained lesser amounts of myristic, palmitoleic, stearic, arachidic, behenic, and lignoceric acids. A fatty acid analysis was also carried out using mixtures derived by saponification of the triglycerides that were obtained by silicic acid chromatography of a chloroform extract. This mixture contained linoleic and linolenic acids in addition to those already mentioned; however, no trace of behenic acid was found. The variance in the relative amount of palmitic acid in this mixture (28.9%) compared to that of the total lipids (90.2%) suggests that large amounts of palmitic acid occur in the pericarps either free as a wax constituent or esterified with other lipid materials.

After the pericarps of the whole fruit had been totally extracted, the hard fruit kernels that remained were ground and extracted with petroleum ether and with chloroform. These oily extracts were found to be totally saponifiable and yielded a mixture of fatty acids qualitatively very similar to that obtained from the triglycerides of the outer portion. The mixture of fatty acids contained, in addition to the components mentioned previously, an unidentified component (28%) having a GLC retention time greater than that of palmitoleic acid and less than that of oleic acid (on polyester columns).

As in the case of Rhus glabra fruit, the green leaves of this plant contained no basic material or steroidal saponins. The ground leaves were extracted successively with petroleum ether, chloroform, ethanol, and

acidified ethanol; a crystalline compound that was isolated from the ethanol extracts was identified as myo-inositol. The petroleum ether and chloroform extracts constituted 8.4 and 4.5 per cent, respectively, of the dried leaves; these extracts consisted of 32 and 34 per cent, respectively, of nonsaponifiable material. Chromatography of the petroleum ether-extractable nonsaponifiable material on alumina yielded three crystalline compounds, IV, VI, and VII.

Compound IV was characterized as a straight chain, primary alcohol on the basis of its infrared and nuclear magnetic resonance spectra. It was further characterized by preparation of the phenylurethane derivative V. Comparison of IV and V with authentic samples led to the assignment of 1-docosanol as compound IV.

Compound VI was obtained as a white, crystalline solid, m.p. 233.3-234.8° (sublimes). The n.m.r. spectrum of VI was not very definitive and the ultraviolet spectrum showed only end absorption. Compound VI showed two bands in the infrared that were attributed to hydroxyl (2.86 μ) and carbonyl (5.83 μ) absorptions. An acetyl derivative (VII) of VI was prepared but could not be obtained crystalline; VI failed to give an oxime or a 2,4-dinitrophenylhydrazine derivative. Mass spectroscopic and osmometric molecular weight determinations of VI yielded values of 456 and 517, respectively. An empirical formula for VI was not established; however, the results of elemental analysis and other data suggest that VI contains 28-32 carbon atoms and 6-7 oxygen atoms.

Silicic acid chromatography of several fractions obtained from alumina yielded VIII, a white crystalline solid that was very nearly pure. Compound VIII was shown to have the formula $C_{29}H_{50}O$. It readily gave an

acetyl derivative and was identified on the basis of its physical properties and GLC retention time as β-sitosterol. GLC analysis of a purified preparation of VIII indicated that this material was a mixture of β-sitosterol (92%), γ-sitosterol (5%), and an unknown (3%).

Side fractions of β-sitosterol (VIII) contained mixtures of several crystalline materials. These mixtures showed strong absorptions at 5.84 μ, but failed to react with 2,4-dinitrophenylhydrazine reagent; they have not been fully investigated.

Another compound (IX), $C_{35}H_{60}O_6$, was isolated during work-up at the saponified chloroform extractives. Compound IX was high-melting and was insoluble in most organic solvents. It readily formed a tetraacetate (X), $C_{43}H_{68}O_{10}$. On the basis of the physical data obtained for IX and X, IX was identified as β-sitosteryl-β-D-glucoside. This assignment was confirmed by comparison of IX and X with authentic samples.

The large scale fractionation of nonsaponifiable leaf material was more conveniently carried out using silicic acid chromatography. Reasonably good separation of several groups of compounds (n-alkanes, terpenoids, fatty alcohols, sterols) was obtained.

GLC analysis of the mixture of n-alkanes indicated the presence of all homologues from C_{14} to C_{33}. Heptacosane ($C_{27}H_{56}$) was present in largest amount; as in the case of the fruit n-alkanes, the C-odd homologues were many times more abundant than those containing an even number of carbon atoms. GLC analysis of the mixture of fatty alcohols that was obtained indicated the following components: 1-docosanol (72.4%), 1-eicosanol (5.7%), 1-tetracosanol (6.7%), and an unknown (15.2%).

A large portion of the nonsaponifiable leaf material was an oily

mixture of ca. six nonvolatile components. This mixture was found to be essentially hydrocarbon in nature, but it was not fractionated successfully.

The relative amounts of the nonsaponifiable leaf constituents were as follows: n-alkanes, 1.0%; nonvolatile constituents, 31%; fatty alcohols, 20%; carbonyl constituents, 0.35%; sterols, 2.1%; IX, 1.8%. Additional quantities of VI were not obtained on scale-up and modification of the isolation procedures.

Qualitatively, the results of GLC analysis of the leaf fatty acids were similar to the results obtained in the investigation of the fruit fatty acids.

CHAPTER I

INTRODUCTION

The Rhus Genus

The common name of this genus, sumac, comes from the Arabic name of the plant, summaq; the scientific family name is Anacardiaceae. There are about 120 species of the genus Rhus and they are most abundant in South Africa. Of the sixteen species found in North America only four are ever trees (1). None of these compares in economic importance with the sumac cultivated in southern Europe. Leaves of these plants contain 25 to 30 per cent of tannic acid, and are regularly gathered, dried, and used in the tanning of fine leathers.

Rhus glabra (smooth sumac), the species investigated here, is familiar as a roadside shrub (1), but occasionally a mature tree can reach a height of 18 to 20 feet. The compound, pinnate leaves contain from 15 to 31 leaflets which are toothed and very whitish beneath. The smooth stems, as well as the leaves, turn bright red in autumn. The flowers are greenish white and grow in compact panicles; the fruit is a compact panicle of velvety drupes that remains erect in winter (2). The fruit, bark, and leaves are used in the treatment of fevers. A cooling beverage, pleasantly acid, is made of the unripe fruit in summer (1).

Staghorn sumac, or Rhus typhina (Rhus hirta, Sudw.), is another species which closely resembles Rhus glabra. This species, the largest of northern sumacs, grows to a height of about 40 feet in the southern part of the United States. Rhus typhina is distinguished from Rhus glabra by having

long, velvety hair on the branches. The fruit, too, is densely hairy (1). Although more common than smooth sumac, staghorn sumac is native nowhere in the South except mountainous regions (3).

Neither of the benign sumacs mentioned above are to be confused with poison sumac (Rhus vernix). Rhus vernix is a tall, rangy shrub growing up to 15 ft. in height. The leaves are compound, but differ from those of the nonpoisonous sumacs by having only seven to eleven leaflets with no teeth on their margins. The fruits are pendant when ripe and are glossy, pale yellow or cream colored with striations on the waxy mesocarp. The distribution of Rhus vernix is from southern Quebec to central Florida; it is found predominantly east of the Mississippi River. The plant is found only in bogs, swamps, and wet bottom lands, whereas benign sumacs never inhabit such wet places. Its toxins have never been identified (4).

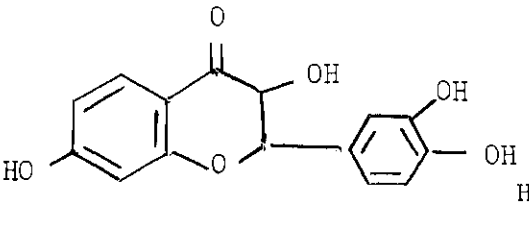
Researches on Rhus Glabra and Related Species

Rhus Glabra

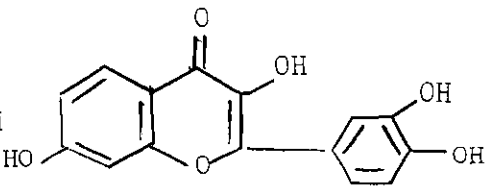
The earliest work of a chemical nature concerning Rhus glabra fruit appears to be that of Cozzens (5) who concluded that "the acid of the sumach is malic, and that it is nearly pure, being only contaminated with a small portion of gallic acid." Rogers (6), however, proved that malic acid existed in sumac as calcium acid malate. Nearly a century later, Sando and Bartlett (7) verified the findings of Rogers (6) that the fruit acid is malic, nearly all in the form of the acid calcium salt. In this study, the malic acid originally isolated as the calcium salt was converted into the silver salt, and the percentage of silver determined. Gallic acid was isolated from the pericarp. In 1925, Peacock and Peacock (8) made an investigation of the tannin of Rhus glabra and reported it to be gallotannic

acid.

It is common knowledge that the plant contains resinous, milky sap; the sap contents of the stems and leaves have been reported to be 0.88 and 0.30 per cent, respectively (9). In 1957, fustin and fisetin were isolated from the heartwood of Rhus glabra (10). Both fustin and fisetin had



fustin



fisetin

been previously isolated from the wood of Rhus succendanea (11). That fustin (3,7,3',4'-tetrahydroxyflavanone) was dihydrofisetin was shown by independent synthesis and by degradation (11).

A pharmacological investigation of Rhus glabra root extract (ethanol) has been carried out (12). In testing the effect of the extract on frog heart, it was noted that direct application produced immediate and marked depression of contractility; also, when the entire vascular system of a pithed frog was perfused with Ringer's solution, injection of a small amount of extract caused a marked decrease in the rate of outflow of solution. At variance with these results, however, was the fact that the Rhus glabra extract produced a decrease in blood pressure in dogs while the heart-rate was unaltered.

The most comprehensive investigation of the fruit of Rhus glabra that was uncovered in a search of the literature is the work of McFadden and

McMurray. The preliminary report (13) contained the results of analyses for a variety of elements and the results of extractions by various solvents, using five pounds of ground fruit. Constants of an oil obtained by petroleum ether extraction were also reported. The complete paper (4) contained, in addition, a report of lipid constituents obtained from the ground fruit. In the complete study, 103 lb. of fruit collected near Columbus, Ohio was extracted with 95 per cent alcohol and gave, after concentration and mechanical removal from the bulk of the extract, 8.87 lb. of crude oil, soluble in petroleum ether. Steam distillation of 1500 g. of crude oil produced only a negligible amount of distillate. Saponification of 1350 g. of purified oil, followed by solvent extraction, yielded 36.19 g. (2.67%) of unsaponifiable material.

A sterol was reported to have been isolated by means of its digitonide from ten grams of nonsaponifiable matter. The sterol (m.p. 137.2°) gave a positive Liebermann-Burchard test for sterols and was further characterized by preparation of an acetyl derivative (m.p. 117-118°). No other information as to its identity was reported. A hydrocarbon (m.p. 68°) isolated from the filtrates after treatment with digitonin was characterized as hentriacontane.

The aqueous portion from saponification of the purified oil yielded 1,267 g. of a mixture of fatty acids; this corresponded to 95.33 per cent of the oil used. Only glycerol (37 g.), amounting to 2.74 per cent of the oil used, was isolated from the aqueous-acid fraction after extraction of the fatty acids. It was characterized by means of the tribenzoate (m.p. 72°). Initial fractionation of the mixture of fatty acids was accomplished through preparation of the lead salts. Regeneration of the acids from the

ether-soluble lead soap, followed by further fractionation and bromination, resulted in the isolation of linoleic acid as the tetrabromide (m.p. 114.5°) and oleic acid as the dibromide.

The ether-insoluble lead soap yielded 184 g. of solid fatty acids. Preparation of the methyl esters, followed by repeated fractional distillation and regeneration of the acids, gave rise to palmitic acid and lignoceric acid. Identifications were based on the melting points, saponification equivalents, and neutralization equivalents of the purified acids and esters. Another acid was tentatively identified as arachidic acid; however, it could not be obtained pure.

Related Species

Numerous reports of investigations of the sumacs in general have appeared in the literature since the early 1800's. Most of these have dealt with the isolation of simple sugars, tannins, and various phenols that occur with the tannin constituents. However, some, particularly those pertaining to Rhus typhina, are sufficiently relevant to the present investigation so as to deserve some mention. One of the more recent reports is that of Plouvier (15) who isolated myo-inositol from Rhus typhina leaves. The ripe fruit of Rhus typhina has been found to contain malic acid (16), gallic acid, and m-digallic acid (17). Gallic acid has also been isolated from Rhus typhina leaf extract (18). This study showed that the amounts of gallic acid and pyrogallol increased and the quantity of tannin decreased on heating or prolonged storage of extracts from various sumacs.

The subject of the fruit constituents of Rhus typhina has been reviewed by Tischer (19). The composition and relative distribution in various parts of the plant were given for the fat, fatty acids, fruit acids

(chiefly malic acid) and the corresponding calcium and potassium salts, gallic acid, ellagic acid, gallotannin (no catechol tannins), pectins, and anthocyanins. The following fatty acids of the fruit wall have been detected by molecular weight determinations and from constants of derivatives: (1) saturated: palmitic, stearic, arachidic, behenic; (2) unsaturated: oleic, elaidic, linoleic, linolenic; (3) hydroxy fatty acids: tetracosanolic ($C_{24}H_{48}O_3$), docosanolic ($C_{22}H_{44}O_3$), eicosanolic ($C_{20}H_{40}O_3$), and octadecanolic ($C_{18}H_{36}O_3$).

The details of the isolation of daucosterin by Tischer (19) also were reported. One gram of crystalline daucosterin (m.p. 305°) was isolated from 4.3 kg. of fruit hairs. The substance gave a positive Liebermann-Burchard test (red-violet, changing to blue, then green). The formula, $C_{35}H_{60}O_6$, was established by elemental analysis. Acid hydrolysis with dilute aqueous-alcoholic hydrochloric acid resulted in the formation of glucose (m.p. 137-138°) and sitosterol as the only degradation products. The sterol gave a correct analysis for sitosterol ($C_{29}H_{50}O$); it was then purified by means of the digitonide, and acetylated to yield a crystalline acetyl derivative (m.p. 126.8-127.2°). A correct analysis for $C_{31}H_{52}O_2$ was obtained. Glucose obtained from acid hydrolysis was further characterized by preparation of the ozazone which showed a melting point of 210° and was not depressed on admixture with authentic glucosazone.

Several members of the Rhus family have been highly regarded for their medicinal value. However, only in one case has a chemical examination of sumac fruit resulted in the isolation of pure compounds having medicinal properties (20). These substances, succedaenin A and succedaenin B isolated from Rhus succedaena Linn, are unsaturated lactones. The formula,

$C_{17}H_{26}O_3$ has been established for succedaenin A; however, a report on the complete structures could not be found.

Purpose of the Investigation

There was ample evidence at the beginning of this investigation that an ethanol extract of the whole fruit of Rhus glabra possessed physiological activity. Therefore an examination of the ethanol extracts of the fruit and leaf portions of the plant for biologically active compounds was planned.

While the isolation of an active principle was an intriguing aspect of this investigation, this pursuit was of secondary importance compared to the primary purpose, which was the isolation and identification of the lipid constituents of the fruit and leaf portions of the plant. A start in this direction has already been made (14). However, it was hoped that with application of modern physical methods, new and/or different compounds could be isolated, and that clarification and superior quantification of the earlier results would be obtained.

CHAPTER II

EXPERIMENTAL

Apparatus and Techniques

In principle, the devices used in the solvent extractions of plant material closely resembled a Soxhlet extractor. The upper and lower portions of these were constructed from five-liter, round-bottomed flasks; a pad of cheese cloth was used in the bottom of the upper portion to minimize the carrying over of undissolved material. Heat was supplied to the extractors by electrically heated mantles of the appropriate size. Petroleum ether (b.p. 30-60°) was always redistilled prior to use. All of the plant material was collected from three locations in the Atlanta, Georgia area. Except when stated otherwise, leaves were collected in mid summer, and the ripe fruit in late autumn. All extractions were carried out on material that had been partially dried by exposure to air for several weeks while spread out in thin layers on Kraft paper. Before extraction with a different solvent, residual solvent in the upper portion of the extractors was removed using a water aspirator. Petroleum ether and chloroform extracts were always dried with anhydrous magnesium sulfate and filtered through paper prior to the removal of solvent. The extracts were first concentrated by distillation at atmospheric pressure; the removal of solvent was completed by drying to constant weight using a rotating evaporator. Crude extracts were stored at room temperature either in glass-stoppered, round-bottomed flasks, or in corked Erlenmeyer flasks.

Unless otherwise stated, all concentrations and evaporations were

performed using a Rinco (Model VE-1000-A) rotating evaporator at water aspirator vacuum and at temperatures below 50°. Drying of solutions and extracts in organic solvents was accomplished by the addition of powdered, anhydrous magnesium sulfate (Mallinckrodt AB 6070) or sodium sulfate (Baker). Before solvent evaporation, the drying agent was removed by gravity filtration and was always washed thoroughly with several portions of solvent.

Melting points, unless otherwise stated, were observed using a Köfler hot stage and are corrected. A Perkin Elmer Model 137 Infracord recording spectrophotometer was used to determine all infrared spectra. Potassium bromide was used for all pellet spectra; pellets were prepared by admixing 0.001 g. of compound and 0.100 g. of potassium bromide using a mortar and pestle. Ultraviolet spectra were determined using a Cary Model 14 recording spectrophotometer. Optical rotations were determined using a Bellingham and Stanley polarimeter (Model No. 397619) equipped with a General Electric Sodium Lab-Arc lamp as the source of the sodium D-line. Each of the reported optical rotations was based on an average of ten replicate measurements. Nuclear magnetic resonance (n.m.r.) spectra were determined using a Varian Model A-60 spectrometer. Tetramethylsilane (TMS) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were used as internal standards. The concentrations of the samples are expressed as weight-volume per cent. A Photovolt (Model 85) Electronic pH meter was employed where pH measurements are stated to have been made using a pH meter; otherwise, these measurements were made using Hydrion paper.

A Mechrolab Vapor Pressure Osmometer (Model 361 A) was used for the determination of molecular weights. Samples were prepared gravimetrically

in glass-stoppered Erlenmeyer flasks. The measurements of ΔR stated represent the results of averaging from three to five replicate measurements. In each determination the instrument was thermostated at 37°; the same lot of solvent was used for the standard solute (benzil) and the unknown solute.

Silicic acid chromatography columns were prepared by mixing the indicated amount of silicic acid (100 mesh, Mallinckrodt AR 2847) with solvent (chloroform, unless stated otherwise). The slurry was placed in a cylindrical column that had a coarse fritted glass disc at the bottom. The column was packed by draining the excess chloroform, accompanied by stirring, followed by vibration until the adsorbent was firm. The packed dimensions are given in the text. Unless stated otherwise, alumina chromatography was performed using Merck acid-washed aluminum oxide. Columns were prepared by filling partially the glass column with solvent, inserting a glass wool plug at the bottom, and depositing a layer of sand (Sea Sand, Will Corporation) over the glass wool. The dry aluminum oxide was slowly passed through a funnel into the column; packing was accomplished in the manner described for silicic acid columns.

Samples were recrystallized several times for microanalysis using glass-stoppered, 15-ml. centrifuge tubes. The hot solutions were centrifuged and the clear supernatant solutions were pipetted into another tube to cool to room temperature; further cooling was carried out by first placing the solutions in the refrigerator, followed by cooling in an ice bath. Each crop of crystalline material was collected on Whatman number 50 filter paper before repetition of the above procedure. Microanalyses were performed by Galbraith Laboratories (Knoxville, Tennessee), Bernhardt Laboratories (Mülheim, West Germany), and Geller Microanalytical Laboratories

(Charleston, West Virginia).

Mass spectra were determined at the University of Illinois by use of an Atlas-MAT Mass Spectrometer CH4. For ionization of the samples a TO 4 thermionic/furnace ion source was used; an Atlas Vacuum lock was used in conjunction with the introduction of samples.

The screening and detection of physiological activity in Rhus glabra extracts was performed by Mead Johnson Research Laboratories, Evansville, Indiana.

Thin-Layer Chromatography (TLC)

All adsorbents and equipment used in conjunction with thin-layer chromatography (TLC) were supplied by Brinkman Instruments, Westbury, New York. The materials and equipment are described in available Brinkman catalogues. Layers of 0.25 mm. thickness for qualitative plates, and 1.00 mm. thickness for preparative plates were spread by means of an adjustable applicator. An Electromechanical Sample Applicator was used in transferring samples to preparative TLC plates. Preparation of aqueous slurries of adsorbents was carried out in accordance with the manufacturer's instructions; the plates prepared in this manner were oven-dried for one to twenty-four hours at 110-120°. After cooling to room temperature, they were stored over indicating silica gel in plastic boxes fitted with grease-sealed lids. Plates stored in this manner could be kept dry from one to three weeks, as indicated by the change in color of the desiccant. When used after extended periods of storage, the plates were reactivated by repeating the oven-drying procedure. Glass plates of dimensions 20 x 20 cm., 10 x 20 cm., and 5 x 20 cm. were used.

Unless otherwise stated, samples were spotted in solution in

chloroform. Where concentrations of samples are not specified, the samples were prepared by transferring a small amount of material to a one-dram vial and dissolving it in 0.1 ml. of solvent delivered by means of a 0.5 ml. syringe. Vertical lines were scored in qualitative layers, both between spots and near the extremities of the layer. Two horizontal lines were drawn in the layer; one near the top of the plate to terminate the travel of the solvent front (unless otherwise stated, a standard traveling distance of 10 cm., measured from the origin, was used), another at a distance of one millimeter from the bottom of the plate. Samples were placed at a height of ca. one inch from the bottom of the plate. Spotting was accomplished by means of commercially made capillaries (2 λ capacity) or by means of drawn melting point capillaries.

TLC plates were developed in glass tanks of the appropriate size lined with paper (Whatman, No. 1). After development, the plates were air-dried to remove solvent. Visualization of spots, except when otherwise stated, was accomplished by placing the dry, developed plate in a covered tank saturated with iodine vapor for five to twenty minutes. For the recording of TLC data, an uncoated glass plate of the appropriate size was placed on top of the visualized plate and the spots, origin, and solvent front were outlined using a grease pencil. The pencil markings were then transcribed on tracing paper along with other necessary information.

Occasionally TLC plates (silicic acid layers only) were prepared by a dipping technique. A slurry was prepared using Silica Gel G, chloroform, and methanol in a weight ratio of 1:2:1. After shaking the ingredients in a wide-necked bottle, small plates (5 x 20 cm.) were easily prepared by dipping, slowly removing the plate at an angle of ca. 45 degrees,

and air-drying for approximately twenty minutes. Layers prepared in this fashion were of good quality, although they were more fragile than those prepared using water.

Gas-Liquid Chromatography (GLC)

All gas-liquid chromatography was performed using a Glowall Corp. Chromalab Model A-110 instrument equipped with a Minneapolis Honeywell continuous recorder. The instrument was used with an argon ionization detector; a Power Proportioning Temperature Programer (Model 240) was employed for the control and monitoring of column temperature. Argon flow rate was not monitored. However, an additional gauge (0-60 p.s.i., Matheson Co., Inc.) was connected to the standard argon regulator for better control of argon inlet pressure. A Gas Chromatography Filter Trap (Matronic Instrument Co., Model XF-100) was spliced into the argon delivery tube between the regulator and chromatograph to dry the gas. Uniformity of column temperature was improved by passing air through an air-bleed system in the column oven walls.

All supports, stationary phases, and prepared column packings were purchased from Applied Science Laboratories, State College, Pennsylvania. Glass columns (6 ft. x 4 mm., unless stated otherwise) shaped in the form of a helix were either purchased from Applied Science Laboratories or constructed locally. (For more detailed specifications of these materials, see Applied Science Catalogue No. 8, October, 1964.)

Chromosorb W (100/120 mesh) used as the support for SE-30 stationary phases was first acid-washed before application of the stationary phase. Approximately 100-200 g. of this support in a one-liter beaker was covered with concentrated hydrochloric acid, stirred occasionally, and

allowed to stand overnight. It was then filtered on a sintered glass funnel, washed free of acid with distilled water, and dried in an oven at 120°. Nitrogen was bubbled through ca. five milliliters of dichlorodimethylsilane in a test tube fitted with a side arm. The exit gas was bubbled through the support by means of a pipette; stirring by use of the pipette was continued for ca. ten minutes. The support was then washed with three portions of methanol and filtered on a sintered glass funnel to remove hydrochloric acid.

SE-30 and DC-QF-1 stationary phases were applied to supports by a "solution coating" technique (21); toluene and acetone (redistilled) were used as solvents. Each support was assumed to have an "absorptivity" of unity (i.e. in the preparation of a 3% SE-30 packing, a solution of 3 g. of SE-30 in 100 ml. of toluene was used). The solution was admixed with 20-25 g. of prepared support. The mixture was swirled in a round-bottomed flask while applying a vacuum with a water aspirator for brief intervals. This was continued until air bubbles could no longer be removed. The coated support was then filtered on a sintered glass funnel using a water aspirator (the product was not washed), and spread out in a thin layer on filter paper to air dry at room temperature. Where toluene was used, the final product was further dried in an oven at 80° to 140° for a short period. The actual per cent of stationary phase on the support was determined by eluting a known amount of the packing with a suitable solvent on a sintered glass funnel and weighing the residue recovered from the filtrate. In one particular case, use of a three per cent solution of SE-30 in toluene resulted in a 2.8 per cent coated support (Chromosorb W).

Prior to packing, the columns were filled with concentrated nitric

acid and were allowed to stand for ten minutes. This was followed by rinsing successively with tap water, distilled water, and methanol. The column was then dried in an oven, cooled to room temperature, and silanized. Silanization was accomplished by filling the column with a one per cent solution of dichlorodimethylsilane in petroleum ether (redistilled, b.p. 30-60°) and allowing it to stand for ca. twenty minutes. It was then drained, rinsed once with methanol, and oven-dried. Glass wool used in conjunction with the columns was silanized using the above solution.

All packing materials, whether purchased or prepared locally, were packed in the same manner. A one-half inch, treated glass wool plug was inserted in the bottom of the column, which was then connected to a water aspirator. The dry support was fed in through the top of the column by means of a glass funnel connected to the column by using a short piece of rubber tubing. With tapping, the column was filled to within one inch of the argon inlet tube and a glass wool plug was inserted.

All columns were cured by programing to a predetermined temperature (detector disconnected, no argon flowing), followed by allowing the column to remain at a somewhat lower temperature overnight with an argon inlet pressure of 10-15 psig, i.e. SE-30 columns were first baked at 300 degrees, then cured at 250°.

All GLC retention times were measured from the solvent front (initial recorder response). Generally, runs at several temperatures and sensitivities had to be made for each sample. Quantification of the chromatograms was performed using a Gelman Instruments Co. (Model 39231) planimeter. The results of quantification by this method for each column were periodically checked using quantitatively prepared standards of several compound

types. This was deemed necessary to insure that the equipment was functioning properly with respect to detector response, recorder damping, and recorder gain. Semi-log plots of carbon number versus log retention time were routinely constructed for GLC of homologous series of compounds (hydrocarbons, fatty alcohols, methyl esters). These were always found to be linear.

Isolation and Identification of Pyrogallol (I)

Solvent Extractions

The fruit was stripped from the stems and sieved through a number 12 sieve to remove larger pieces of extraneous plant material. The fruit was ground in a ball mill and sieved through a finer mesh sieve.

The 3.0 kg. of red-brown powder resulting from the above treatment was placed in two modified Soxhlet extractors. When the powder was initially extracted with petroleum ether (b.p. 30-60°), it was noted that the solution did not siphon from the upper portion of the extractors at a rate fast enough to prevent an over abundance of solvent from accumulating. This difficulty was alleviated somewhat by removal of the powder from the extractors and admixing it with Celite (Johns-Manville, used as received).

Extraction with petroleum ether was continued for two days, which produced 447 g. of a brown, viscous oil after removal of solvent. The powder was next extracted with chloroform for two days and gave an additional 44.9 g. of oil. The chloroform extraction was followed by extraction with 95% ethanol for two days. After removal of the ethanol by distillation at atmosphere pressure, this extract was made up to a volume of 2.0 l. with water and produced a red, sweet-smelling solution.

The aqueous solution was made acidic (pH 1.5, checked with a pH

meter) by the addition of concentrated hydrochloric acid and extracted continuously for a total of 32 days with chloroform in a liquid-liquid extractor. Chloroform fractions were taken ca. every three days. These solutions were cooled in an ice bath and filtered through paper to remove small amounts of chloroform-insoluble material (2-4% by weight). Removal of chloroform from the filtrates produced a total of 220.5 g. of black tar.

To the acidic, aqueous residue was added 1400 ml. of concentrated ammonium hydroxide. The resulting solution was extracted continuously with chloroform for a total of 67 days. Fractions were taken ca. every five days and the chloroform was removed as before. This treatment yielded 17.1 g. of black tar. The facile sublimation of a portion of this extract was noted by the appearance of a small amount of white crystalline solid on the walls of the flasks during the removal of solvent.

Isolation of I

The solubility of small amounts of the extract obtained by chloroform extraction after the addition of ammonium hydroxide was tested in several common solvents. It was found to be soluble at room temperature in water, ethanol, and methanol, but essentially insoluble in boiling benzene and cyclohexane. It was found that when 1.5554 g. of the crude extract was triturated with boiling chloroform-benzene (1:1) and the hot solution was filtered through a fluted paper funnel and gradually cooled to -80°C in dry ice-acetone, a small amount of a white, crystalline solid resulted. Some additional crystalline material was obtained by applying the same procedure to the undissolved extract. The solid from both treatments was collected on a small Buchner funnel and dried for one hour in vacuo at 78° and gave 0.2118 g. of I, an off-white, crystalline solid, m.p.

129-132°.

Additional crystalline material was obtained more conveniently by vacuum sublimation; thus 8.1 g. of crude tar was sublimed in several portions at 95-105° and 0.5-2.0 mm. pressure. A representative fraction showed m.p. 127-132°. The solid obtained initially from chloroform-benzene was combined with the sublimates (2.8925 g. total) and yielded after crystallization from chloroform-benzene, 1.5724 g. of white plates, m.p. 128-131°. Since the compound slowly sublimed from the hot stage on melting, its melting point was determined in a sealed capillary using a modified Hershberg apparatus; m.p. 125-127°. A small amount of this material was recrystallized twice from chloroform for analysis.

Anal.	$C_6H_6O_3$	Calc'd: C, 57.14; H, 4.86
	(126.11)	Found : C, 56.58; H, 5.25

Physical and Chemical Evidence for Identification

In order to determine the molecular weight of I in absolute ethanol using a vapor pressure osmometer, a calibration curve for that solvent first had to be obtained. For this purpose, nine solutions of benzil (recrystallized several times from ethanol) were made up so as to provide solutions ranging in concentration from 0.0024 molal to 0.1363 molal. The solutions were prepared gravimetrically in 10-ml. Erlenmeyer flasks fitted with ground glass stoppers. Readings of ΔR values were recorded by taking replicate measurements as before and the concentrations were determined from the calibration curve. The results are given below.

The molecular weight calculated by averaging the values given below was found to be 124.1 (theory for $C_6H_6O_3$ = 126.11).

A small amount of I was dissolved in 1 ml. of absolute ethanol.

Sample No.	ΔR	Molality (x 10^2)	Molecular Weight
(a)	2.78	1.62	133.4
(b)	5.56	3.43	128.5
(c)	8.49	5.46	123.0
(d)	11.48	7.74	111.3

The addition of one drop of 1% ferric chloride solution (methanol) produced a dark green coloration immediately. After two days, the color changed to dark orange. Authentic pyrogallol (Eastman, used as received) was found to undergo identical color changes under the same conditions.

The infrared spectrum of I exhibited λ_{max} 2.59, 2.66, 3.27, 4.12, 6.18, 6.59, and 7.13 μ (saturated, in chloroform). The infrared spectrum of authentic pyrogallol was recorded and found to be identical to that of I.

The nuclear magnetic resonance spectrum of I, determined in deuterium oxide solution, revealed absorptions at 5.33 τ (HDO) and ca. 3.3 τ; the low-field absorption appeared to be a multiplet consisting of seven lines. The n.m.r. spectrum of authentic pyrogallol determined under similar conditions was superimposable with that of I.

Preparation of Pyrogallol Triacetate

To 0.1603 g. of I in a 10-ml. round-bottomed flask was added 5.0 ml. of acetic anhydride (Eastman, used as received) and eight drops of pyridine (Baker, used as received). The flask was fitted with a reflux condenser and warmed on a steam bath for 1-3/4 hours. Three milliliters of water was added to the solution after cooling in an ice-salt bath. This resulted in the formation of a white crystalline solid which was collected, rinsed with a few drops of 95% ethanol, and air dried. This gave 0.2356 g.

(74% based on $C_6H_6O_3$) of a triacetyl derivative, m.p. 161-164°. A small amount was twice recrystallized for analysis with no improvement of melting point. The analytical sample was dried overnight at 59° in vacuo.

Anal.	$C_{12}H_{12}O_3$	Calc'd:	C, 57.14; H, 4.80
	(252.22)		C, 57.15; H, 5.04

The infrared spectrum showed λ_{max} 5.60 μ (10% in chloroform).

Search for a Basic Fraction in Autumnal Leaf Extract

The leaves were gathered in late October, 1963. Only leaves which had mostly turned garnet were collected. After the leaves had air-dried for several weeks, they were pulverized in a ball mill to a fine, garnet-colored powder.

The above procedure yielded a total of 1,076 g. of powder; 180.5 g. of this was extracted continuously for three days in a Soxhlet extractor with methanol. The methanol was removed; the extract was then dried in vacuo for several hours with occasional warming on a steam bath. This yielded 89.6 g. of a dark brown glass.

Most of the above extract (80.7 g.) was redissolved in 150 ml. of methanol. The resulting solution was then made acidic by the addition of 250 ml. of five per cent acetic acid and extracted four times with 100-ml. portions of n-hexane (Phillips, redistilled). The n-hexane solutions were dried with anhydrous magnesium sulfate, filtered, and stripped of solvent. A total of 10.8 g. of a brown syrup was recovered from the n-hexane extracts.

The acidic aqueous solution was divided into two parts and both parts were treated in the following manner. The solutions were cooled to 5° and were made basic (pH 10) with concentrated ammonium hydroxide

(followed by use of a pH meter). After the addition of base, a brown, gummy solid, which had begun to precipitate at ca. pH 8 was collected by filtration and washed with several milliliters of chloroform. The precipitate could not be redissolved in 5% acetic acid and was not soluble even after the addition of two milliliters of concentrated hydrochloric acid; it was discarded.

The filtrate was extracted with four 100-ml. portions of chloroform and the chloroform solutions were combined and concentrated to a volume of ca. 200 ml. The resulting solution was extracted several times with small portions of five per cent acetic acid. The acetic acid solutions were combined, made basic (pH 10) by the addition of ammonium hydroxide, and extracted several times with chloroform. The chloroform solutions were combined, dried, and gave, after removal of solvent, only a trace of a yellow, viscous liquid. It was not examined further.

Hemolytic Test for Steroidal Saponins

Blood Standardization

For the purpose of conducting hemolytic tests for steroidal saponins, ca. 20 ml. of my own blood (type O-negative) was drawn at the Georgia Institute of Technology Infirmary. The whole blood was immediately transferred to a Nalgene bottle containing 100 ml. of a 0.85 per cent aqueous sodium chloride solution prepared by dissolving 8.58 g. of sodium chloride (Baker reagent grade, used as received) in one liter of distilled water.

A stock suspension of red blood corpuscles was prepared and standardized in accordance with a previously established procedure (22). The above suspension was divided into four equal portions, and each portion

was centrifuged in a 40-ml. centrifuge tube. The supernatant solution from each sample was removed by means of a pipette and the red corpuscles were washed by centrifuging each sample with two 25-ml. portions of 0.85 per cent aqueous sodium chloride. The red corpuscles were then combined and suspended in 400 ml. of the saline solution in a screw-top, Nalgene bottle. The bottle was stored in a refrigerator when it was not being used.

Some of the turbid suspension was standardized using digitonin (CalBioChem, used as received), a known plant saponin. A digitonin solution was prepared by dissolving 10.3 mg. in 100 ml. of 80 per cent ethanol. One milliliter of this solution was placed in a 12-ml. conical centrifuge tube along with 10 ml. of the stock red corpuscle suspension. This sample and a blank (digitonin excluded) were shaken separately and were allowed to stand at room temperature for five minutes. Both solutions remained turbid and, when centrifuged, yielded a blood-red precipitate. A 25-ml. aliquot of the stock corpuscle solution was then successively diluted with 0.85 per cent sodium chloride solution such that 10 ml. of the resulting standard solution was completely clarified in less than five minutes by the addition of one milliliter of digitonin solution. Centrifugation of this hemolyzed solution did not produce a precipitate, whereas a definite red corpuscle precipitate resulted using a blank. The standardized red corpuscle suspension was used in the testing of Rhus glabra ethanol extracts; it was stored in a screw-cap Nalgene bottle in a refrigerator when not in use.

Testing of Rhus Glabra Extracts

A 166.2 g. sample of powdered green leaves (collected 7-23-63) was

extracted successively with petroleum ether, chloroform, and 95 per cent ethanol. One year and two months later, a small sample of the ethanol extract was tested for hemolytic activity. A 555 mg. sample of ethanol-free extract was dissolved in 100 ml. of 80 per cent ethanol. A 1-ml. aliquot of this solution was found not to clarify 10 ml. of standardized red corpuscle solution; instead, centrifugation after a period of five minutes yielded a brown precipitate. Identical negative results were obtained when two milliliters of the ethanol solution was admixed with five milliliters of red corpuscle solution.

From a 1750 g. sample of whole fruit (collected in early November, 1962) there was obtained by chloroform extraction (four days) 137.2 g. of a dark, viscous oil. Continued extraction with 95% ethanol (35 days) produced an additional 183.6 g. of extract of the same description. An ethanol solution was prepared using 664 mg. of the ethanol extract and 100 ml. of 80 per cent ethanol. The hemolytic testing was carried out in the same manner described for the leaf extract; the results, too, were identical.

Lipids from the Outer Portion of the Fruit

Preliminary Investigation

Solvent extraction of the whole fruit (3-4 kg.) was first carried out using petroleum ether (b.p. 30-60°). After extraction for several days, the extract was concentrated, dried with anhydrous magnesium sulfate, and evaporated; the magnesium sulfate used to dry the extract was eluted with chloroform to recover a significant amount of adsorbed extractives. The fruit was then extracted with chloroform for several days. This extract was treated in the same manner, with the exception

that absolute ethanol was used to recover material which had been adsorbed by the magnesium sulfate. Solvent extraction of the same fruit was then continued with 95 per cent ethanol, and finally with 70 per cent ethanol which had been made 0.2 N in hydrochloric acid. Neither ethanol extract was weighed, but were combined with similar materials from subsequent extractions. The last extraction was accomplished by stirring the fruit with hot, acidified ethanol in a 12-l. round-bottomed flask. Magnesium sulfate washings were kept separate from the main portions of the extracts. The results of extraction of several portions of fruit are given below (% refers to the total weight of each extract relative to the weight of partially dried fruit). All of the extracts were obtained as dark, viscous

Weight of Whole Fruit	Petroleum Ether Extract			Chloroform Extract		
	Bulk	Washings	%	Bulk	Washings	%
3,200 g.	118.7 g.	24.5 g.	4.5	59.7 g.	17.2 g.	2.4
3,200	193.4	12.1	6.4	70.4	12.5	2.6
3,200	129.6	15.4	4.5	64.7	26.5	2.9
3,200	99.3	36.9	4.3	54.7	13.6	2.1
3,600	131.6	19.6	4.2	60.2	15.4	2.1
3,600	121.1	24.2	4.0	57.7	21.5	2.2
3,740	113.8	30.5	3.9	62.0	15.7	2.1

oils.

Silicic acid chromatography was next carried out using 35.04 g. of chloroform extract (column dimensions: d = 6.3 cm., h = 76.2 cm.). The extract was dissolved in an equal volume of chloroform and the resulting solution pipetted on to the top of the column. This was followed by washing the material on the column with several 5-ml. portions of chloroform

while draining solvent from the bottom of the column. A 4-l. separatory funnel was used as a solvent reservoir. The column was allowed to operate for three days using an automatic fraction collector; ca. 20-ml. fractions were taken. As the quantity of material being eluted became smaller, the solvent in the reservoir was successively changed to two per cent, then twenty per cent methanol (stock) in chloroform. The contents of the test tubes were combined to yield fractions comprising 10-15 of the fractions originally collected.

Twenty of the fractions which resulted represented all of the material that was eluted with chloroform. The bulk of this material was contained in fractions 4-6 (18.8017 g.). A total of 33.7009 g. (96%, representing 41 fractions) was recovered from the column. Attempts were made to crystallize several of the fractions using a variety of organic solvents and solvent pairs. Neither a crystalline solid nor a well-defined precipitate was ever obtained. The n.m.r. spectra of fractions four and five were recorded. The results, whether using the neat liquids or 15 per cent carbon tetrachloride solutions were essentially the same for the four samples; vis. significant absorptions were present at 9.09 (weak) and 8.73 τ (strong), and 5.79 and 4.64 τ (both weak, resembling poorly-defined triplets).

Petroleum Ether Extract

Nonsaponifiable Portion. A petroleum ether extract (121.1 g.) was saponified using a solution containing 84 g. (1.50 moles) of potassium hydroxide dissolved in 300 ml. of 50 per cent aqueous methanol. The reaction was carried out by heating the mixture in a 1-l. round-bottomed flask under reflux using a steam bath. After 24 hr., the mixture was

cooled, diluted with 100 ml. of water, and extracted continuously overnight with petroleum ether (redistilled, b.p. 30-60°). Since the petroleum ether solution in the receiving flask was badly emulsified with water, it was washed repeatedly with 200-ml. portions of water in a separatory funnel; the aqueous washings were discarded. The petroleum ether solution was dried overnight with anhydrous sodium sulfate, filtered, and the petroleum ether was evaporated. The nonsaponifiable material (0.4379 g.) which remained was a pale yellow, waxy solid.

The contents of the 1-l. flask (basic aqueous-methanolic layer plus supernatant petroleum ether) was transferred to a 2-l. round-bottomed flask, diluted with 300 ml. of water, and extracted continuously with petroleum ether for five days. Drying and removal of the solvent from this extract as before yielded only a negligible quantity of material. The petroleum ether in the extraction flask was siphoned off, discarded, and continuous extraction of the basic solution was resumed using diethyl ether. After three days extraction, an appreciable amount of soap had accumulated in the receiving flask; the extract was washed free of soap as previously described before drying and removal of solvent. In order to prevent further emulsification, the contents of the extraction flask were transferred to a 3-l. round-bottomed flask and the mixture was diluted with water to a total volume of ca. 2.5 l. The data for the individual extracts that resulted from continuous extraction with petroleum ether and diethyl ether are summarized below.

All of the fractions were orange, waxy solids having pleasant aromas.

Fraction No.	Solvent	Duration (days)	Weight (g.)
1	petroleum ether	3	0.4379
2	" "	5	trace
3	diethyl ether	4	4.5325
4	" "	2	2.4887
5	" "	3	0.3600
6	" "	3	0.2892
7	" "	3	0.0560

Chromatographic separations. The diethyl ether extracts, fractions 3-7, were combined (7.7264 g.) and chromatographed on 400 g. of Unisil silicic acid (column dimensions: d = 4.8 cm., h = 43 cm.). Using an automatic fraction collector, 22 fractions, each representing ca. 200 ml. of effluent, were taken using chloroform as the eluting solvent (77% recovery of starting material). Further elution with 700 ml. of 50 per cent methanol-chloroform yielded only a negligible amount of material; however, when the adsorbent was unpacked and exhaustively washed on a sintered glass funnel with boiling acetone, a small amount of black tar was obtained. The mixture was dissolved in acetone, decolorized using charcoal (Darco, used as received), and filtered with suction on a bed of celite. Slow evaporation of the solvent yielded a trace of white crystalline solid, m.p. 180.5-183.5°; it was not examined further.

Fractions two and three, 2.6558 g. and 0.9370 g., respectively, were combined and a small amount of this material was subjected to infrared analysis; absorptions were present at 3.40 and 6.83 μ (10% in chloroform) in addition to those attributed to chloroform. The remainder of

the material, ca. 3.5 g., was chromatographed on 241 g. of aluminum oxide (column dimensions: d = 1.8 cm., h = 115 cm.). Fifty-milliliter fractions were taken (some mechanical loss during manipulation) using petroleum ether as the eluting solvent. Fractions 3-7, after removal of solvent, were white crystalline solids. The weights and melting points are given below. The n.m.r. spectrum of fraction four showed only absorptions

Fraction No.	Weight (g.)	m.p.
1,2	------	------
3	0.1797	57-59°
4	2.0210	58-62°
5	0.1329	------
6	0.0176	56-65°
7	0.0056	------

at 8.75 and 9.12 τ (weak) (10% in deuteriochloroform). Further elution with benzene (freshly distilled) yielded a negligible amount of material.

Gas-liquid chromatography. Preliminary GLC experiments indicated that the petroleum ether fractions from alumina chromatography (above) were complex mixtures. For the purpose of identifying these constituents by GLC, a number of hydrocarbon standards first had to be prepared.

n-Octacosane, n-triacontane, n-dotriacontane, and n-hexatriacontane were prepared by Wurtz reactions using the appropriate alkyl bromides (Eastman). The general procedure was the same in each case. The reactions were carried out in a 250-ml., three-necked, round-bottomed flask fitted with a reflux condenser equipped with a calcium chloride drying

tube, a dropping funnel, and a glass stopper. Sodium metal was cut under benzene into small pieces and placed in the flask (the flask had been heated strongly to expel water vapor and then allowed to cool somewhat before the addition of sodium). A small amount of the alkyl bromide was delivered from the funnel into the flask and the reaction was started by passing a flame under the bottom of the flask. The rate of addition of alkyl bromide was adjusted so that the reaction sustained itself. Frequently air had to be blown over the flask to slow the rate of reaction. After the last addition of alkyl bromide (20.0 g. total) the flask was warmed gently and the contents were stirred occasionally. Excess sodium was destroyed by the successive addition of absolute ethanol, ethanol-water mixtures, and water. The hydrocarbon was extracted with chloroform, and the chloroform solutions were dried with anhydrous magnesium sulfate and evaporated to dryness. The crude product was recrystallized several times from redistilled n-hexane.

The yields of purified hydrocarbons and other pertinent data are given below.

n-Alkane	Alkyl Bromide	% Excess Na	Yield	m.p.	Lit. m.p. (23)
C_{28}	1-Bromotetradecane	500	2.7 g. (20%)	63-63.5°	60°
C_{30}	1-Bromodecane	89	4.8 g. (33%)	65-66°	61.1°
C_{32}	1-Bromohexadecane	233	6.0 g. (35%)	71-71.5°	70.5°
C_{36}	1-Bromooctadecane	114	4.8 g. (32%)	75-77°	76°

Another hydrocarbon, n-tricosane, was prepared by catalytic hydrogenation of 11-tricosene. Fifty grams (0.18 mole) of 11-tricosene (Matheson,

Coleman and Bell, technical, m.p. 1-3°) was dissolved in 100 ml. of absolute ethanol in a hydrogenation bottle. The bottle was flushed with nitrogen, and 20 g. of catalyst (5% Pt on carbon, Engelhard) was added. The hydrogenation was carried out at 40 psig and ca. 28°; at the end of twenty hours, the pressure remained constant at 26.5 psig, which indicated an uptake of ca. 0.17 mole of hydrogen. The product, which did not appear to be appreciably soluble in ethanol, and catalyst were collected on a Celite filter bed on a sintered glass funnel. The catalyst-hydrocarbon mixture was washed with a total of 500 ml. of boiling acetone, followed by washing with 500 ml. of boiling benzene (redistilled, stored over sodium). Removal of acetone from the acetone washes yielded only a trace of product, which was discarded. The benzene filtrates were combined and gave, after removal of benzene, 42 g. (72%) of crude tricosane. After one recrystallization from petroleum ether the pure n-tricosane (24 g., 41%) melted at 47.8-48.1°.

The sources of other n-alkane standards which were used are cited below; they were used as received. Two standard mixtures were used to

Carbon Nos.	Source
$C_{20,22}$	Matheson, Coleman and Bell
C_{24}	Applied Science Laboratories
$C_{26,28}$	Petroleum Institute, Penn. State Univ.
$C_{10,12,14,16,18}$	(Mixture K-202) Applied Science Laboratories

establish the retention times of the various n-alkanes; one was Applied

Science Laboratories mixture K-202. The other was prepared by dissolving 10 mg. of each of the remaining n-alkanes ($C_{20,22,23,24,26,28,30,32,36}$) in 0.5 ml. of petroleum ether and combining the individual solutions. Generally, from 0.5-1.0 µl. of one per cent (weight/volume) solutions were injected into the chromatograph. Several determinations at various temperatures and sensitivities were made to facilitate the comparisons of retention times between unknowns and standards. Gas-liquid chromatography of material from fraction number four (cf. p. 28 above) was performed using an SE-30 column (3% SE-30 on Chromosorb W). The assignments and quantification of the n-alkane constituents are given below in Table 1.

Fatty Acids. A portion of the basic, aqueous layer, after saponification and extraction of unsaponifiable material was cooled in ice and made acidic (pH 1) by slow addition of concentrated hydrochloric acid. The acidic solution was extracted with several portions of diethyl ether; these extracts were combined, dried overnight with anhydrous magnesium sulfate, and evaporated to dryness. This yielded 84.3 g. of a brown oil.

Basically, the procedure used for the preparation of methyl esters was that of N. S. Radin and coworkers (24). A 30-mg. portion of the above material was dissolved in one milliliter of methanol in a 15-ml. centrifuge tube. To the resulting solution and undissolved material was added 50 µl. of concentrated hydrochloric acid and 0.2 ml. of 2,2-dimethoxypropane (Dow Chemical Company, redistilled, b.p. 66-70° at 738.8 mm.). The centrifuge tube was stoppered and the esterification was allowed to proceed for two hours. The solution (only a trace of undissolved material remained) was extracted with three 5-ml. portions of petroleum ether (redistilled, b.p. 30-60°). These extracts were

Table 1.[a] n-Alkanes in Petroleum Ether Fruit Extract.

n-Alkane	Retention Time (t_R) t = 240°	t = 210°	t = 155°	% Total Area
C_{15}			2.3	trace
C_{16}			3.8	trace
C_{17}		1.1	5.9	trace
C_{18}		1.5	9.0	trace
C_{19}		2.1		trace
C_{20}		2.9		trace
C_{21}		4.0		trace
C_{22}	4.2	5.5		trace
C_{23}	5.5	7.5		1.2
C_{24}	7.3			0.3
C_{25}	9.4			3.8
C_{26}	12.3			0.4
C_{27}	16.0			25.6
C_{28}	20.7			2.1
C_{29}	27.2			52.2
C_{30}	34.9			1.6
C_{31}	45.4			10.5
C_{32}	58.8			0.9
C_{33}	76.0			1.5

a. C_{15}-C_{22} were not considered in calculating the total area; trace = $< 0.3\%$; p_i = 15 psig.

combined, dried over anhydrous magnesium sulfate, evaporated to dryness, and gave 25.6 mg. of a yellow oil. The methyl esters were redissolved in petroleum ether and analyzed (GLC); the results are given in Table 2.

Table 2. GLC of Methyl Esters from Whole Fruit - Petroleum Ether Extract.

Methyl Ester		Retention Time (t_R) EGSS-X[a]	EGS[b]	SE-30[c]	% Total Area
C_{14}	Myristate	2.1	4.8		1.7
C_{16}	Palmitate	3.7	8.3		41.8
C_{16}	(-2H) Palmitoleate	4.6	10.2		1.9
C_{18}	Stearate	6.5	14.3		10.7
C_{18}	(-2H) Oleate	7.7	17.1		16.1
C_{20}	Arachidate	11.3	25.0	3.5	18.5
C_{22}	Behenate	19.7		6.0	9.3

a. 11% EGSS-X on 100/120 mesh Chromosorb W; t = 177°, p_i = 22 psig.

b. 11% EGS on 100/120 mesh Chromosorb W; L = 12 ft.; t = 183°, p_i = 25 psig.

c. 3% SE-30 on 100/120 mesh Chromosorb W; t = 227°, p_i = 20 psig; t_R's are relative to n-hexane vice petroleum ether.

Chloroform Extract

Nonsaponifiable Portion. The bulk of a chloroform extract (59.7 g.) was saponified in a 500-ml. round-bottomed flask with a solution containing 15 g. (0.29 mole) of potassium hydroxide in 100 ml. of 50 per

cent aqueous methanol. The mixture was heated on a steam bath under reflux for 24 hr., cooled to room temperature, and extracted several times with petroleum ether (total of 500 ml.). The petroleum ether extracts were combined, dried with magnesium sulfate, and evaporated to dryness. Only a small amount (0.2325 g.) of a dark, viscous oil was obtained; it was not examined further.

Fatty Acids. The fatty acid constituents were isolated from the saponification reaction mixture using the same procedure previously described for the petroleum ether extractives. In this case, the methyl esters were prepared using diazomethane. The diazomethane, prepared by the reaction of N-methyl-N-nitrosourea with potassium hydroxide (25), was not distilled.

A portion of the mixture of fatty acids (0.8 g.) was dissolved in a small amount of diethyl ether in a 50-ml. Erlenmeyer flask. The flask was cooled to five degrees and the ethereal diazomethane solution was added slowly until nitrogen was no longer evolved; 10 ml. was then added in excess. The excess diazomethane was allowed to evaporate at room temperature. The ether solution that remained was diluted with ether, dried with anhydrous magnesium sulfate, evaporated to dryness, and gave 0.8084 g. of a yellow oil. The results of the GLC analysis are given in Table 3.

Fatty Acids from Chromatographically Separated Triglycerides*

Extraction and Purification of Crude Lipids. Chloroform extraction of 3,500 g. of whole fruit (not previously extracted with petroleum

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* The author is very grateful to Mr. P. A. Burns for performing the isolation work described in this section.

Table 3. GLC of Methyl Esters from Whole Fruit - Chloroform Extract.

Methyl Ester		Retention Time (t_R) EGSS-X[a]	EGS[b]	SE-30[c]	% Total Area
C_{14}	Myristate	1.4	4.2		0.1
	Unknown	2.0	3.0		trace
C_{16}	Palmitate	2.4	7.3		90.2
C_{16}	(-2H) Palmitoleate	2.9	9.0		0.4
	Unknown	3.5	11.8		0.3
C_{18}	Stearate	4.1	12.6		1.2
C_{18}	(-2H) Oleate	4.9	15.0		5.0
	Unknown	5.9	19.9		0.2
C_{20}	Arachidate	7.1	26.6	3.5	0.5
	Unknown	8.4		5.7	1.2
C_{22}	Behenate	12.2		6.0	0.1
C_{24}	Lignocerate	20.5		11.1	0.6

a. 11% EGSS-X on 100/120 mesh Chromosorb W; t = 184°, p_i = 25 psig.

b. 11% EGS on 100/120 mesh Chromosorb W; L = 12 ft.; t = 188°, p_i = 25 psig.

c. 3% SE-30 on 100/120 mesh Chromosorb W; t = 227°, p_i = 20 psig; t_R's are relative to n-hexane vice petroleum ether.

ether) for four days yielded 296.0 g. of a dark, viscous oil. Ten grams of the crude material was dissolved in 100 ml. of chloroform-methanol (2:1; CH_3OH was redistilled from magnesium) and shaken with 25 ml. of distilled water in a 250-ml. separatory funnel. The heavy emulsion that resulted was broken only after several hours of equilibration of the two phases. The bottom phase was separated, transferred to another separatory funnel, and shaken with 25 ml. of upper phase from the equilibration of a chloroform-methanol-water mixture (14:7:5). After the two phases had separated, the bottom layer was removed and again distributed in 25 ml. of upper phase from the chloroform-methanol-water mixture. The bottom phase from this distribution was dried and evaporated; this gave 8.3091 g. of crude lipids. The upper phases from the preceding separations were combined, and gave, after evaporation of solvent at 74°, 0.4268 g. of a black gum.

For further purification of the crude lipids, a Unisil silicic acid column was prepared using n-pentane (column dimensions: d = 5 cm., h = 45 cm.). Fractions (15-20 ml.) were taken using an automatic fraction collector. The weights of fractions and sequence of eluting solvents are given in Table 4. A total of 7.5914 g. (91%) was recovered from chromatography.

Fatty Acids. Fractions 242-315 (3.3170 g.) were combined and saponified using a solution containing 2.5 g. of sodium hydroxide in 50 ml. of 50 per cent aqueous methanol (steam-bath temperature, for 24 hr.). The cooled mixture was diluted with 50 ml. of water and extracted with 150 ml. (3 x 50 ml.) of petroleum ether. After drying with magnesium sulfate and evaporation of solvent, this extract yielded 25 mg. of

Table 4. Unisil Chromatography of Unsaponified Fruit Lipids.

Solvent	Volume (l.)	Fraction No.	Weight (g.)
Petroleum Ether	0.90	1-60	0.1217
6% C_6H_6 in Petroleum Ether	0.91	61-129	trace
20% C_6H_6 " " "	2.02	130-241	0.0586
65% C_6H_6 " " "	2.34	242-371	3.2660
C_6H_6	1.68	372-464	1.1094
$CHCl_3$	4.85	465-473	0.6992
2% CH_3OH in $CHCl_3$	3.28	474-915	1.9629
10% CH_3OH " "	1.48	916-997	0.2536
CH_3OH	0.45	-------	0.1200

nonsaponifiable material; it was not investigated. Concentrated hydrochloric acid was added to the aqueous-methanolic portion until a pH of one was established. The acidic solution was extracted with 150 ml. of petroleum ether, followed by extraction with two 50-ml. portions; the extracts were combined, dried with magnesium sulfate, and yielded 1.9471 g. of fatty acids after evaporation of solvent. An absorption at λ_{max} 5.82 μ (6%, in chloroform) was present in the infrared spectrum.

The preparation of methyl esters of the above mixture was performed on a small scale by dissolving 30 mg. of the fatty acids in a mixture of one milliliter of dry methanol, 0.2 ml. of 2,2-dimethoxypropane, and 50 μl. of concentrated hydrochloric acid in a 15-ml. centrifuge tube.

After several minutes, a clear solution resulted; the reaction was carried out at room temperature for 2 1/4 hr. At the end of this time, the solution was extracted three times with 5-ml. portions of petroleum ether. These extracts were combined, dried with anhydrous sodium sulfate, and gave, after evaporation of solvent, 31.1 mg. of a colorless oil (II). It showed λ_{max} 5.72 μ (6%, in chloroform).

Several of the benzene fractions (cf. Table 4) that had been combined (fraction No. 388-397, 0.1581 g.) were saponified in accordance with the procedures previously described. Prior to saponification this material showed absorptions in the infrared at λ_{max} 3.33, 3.42, 5.69, 6.81, 7.26, and 8.60 μ (liquid film). Saponification yielded in addition to a small portion of nonsaponifiable material which was discarded, 0.0977 g. of fatty acids (λ_{max} 5.82 μ, 3% in carbon tetrachloride). Thirty milligrams of this mixture was esterified under conditions identical to those given above and gave 28.5 mg. of a colorless oil (III); it showed λ_{max} 5.72 μ (liquid film).

Gas-liquid chromatography was carried out using petroleum ether solutions containing small amounts of (II) and (III); i.e. 1% (w/v). The GLC standards (supplied as mixtures) which were used to establish the retention times (t_R) of the various methyl esters were also determined in petroleum ether solution at about the same concentration. Several determinations of retention times for the constituents of (II) and (III) were made on three columns using various combinations of p_i, t, sample size, and sensitivity. The data which were obtained are given in Table 5.

Table 5. GLC of Methyl Esters Derived from Fruit Triglycerides.

Methyl Ester		t_R (EGA)[a]		t_R (EGS)[b]		t_R (EGSS-X)		% Total Area	
		II	III	II	III	II[c]	III[d]	II	III
C_{14}	Myristate		3.0		4.7	1.2	1.2	trace	0.4
C_{16}	Palmitate	5.4	5.5	7.7	7.8	2.0	1.9	28.9	13.1
C_{16}	(-2H) Palmitoleate		6.2	9.5	9.5	2.9	2.8	0.3	2.6
C_{18}	Stearate	10.1		12.8	12.9	3.3	3.2	0.7	3.4
C_{18}	Oleate	11.2	11.2	15.2	15.5	4.0	3.8	65.2	37.3
C_{18}	(-4H) Linoleate	13.4	13.4	19.5	19.7	4.8	4.7	2.3	3.3
C_{20}	Arachidate					5.6	5.5	0.1	2.8
C_{18}	(-6H) Linolenate					7.0	7.0	0.1	4.6
	Unknown					9.0		0.3	
C_{24}	Lignocerate					15.8	15.6	0.7	20.5
	Unknown					17.1	16.7	0.7	12.1
	Unknown					34.3		0.1	
	Unknown					39.6		0.7	

a. 15% EGA on 100/120 mesh Chromosorb W; t = 190°, p_i = 20 psig.

b. 11% EGS on 100/120 mesh Chromosorb W; L = 12 ft.; t = 190°, p_i = 20 psig.

c. 11% EGSS-X on 100/120 mesh Chromosorb W; t = 190°, p_i = 24 psig; retention times are relative to n-hexane vice petroleum ether.

d. Ibid.; t = 193°.

Investigation of the Inner Portion of the Fruit

Solvent Extractions

After the whole fruit had been extracted successively with petroleum ether, chloroform, ethanol, and acidified ethanol, the pericarps, or outer portions, appeared to have been totally extracted. The hard fruit kernels which remained were air-dried, separated from residual outer material, and ground in a meat grinder; this produced a coarse powder. A total of 3,909 g. of powder was extracted with petroleum ether in two extractors for a period of five days. This yielded, after removal of solvent, 225.6 g. of a yellow, viscous oil. The extractions were continued using chloroform for a period of four days and yielded an additional 35.9 g. of oil.

Nonsaponifiable Portions

Petroleum Ether Extracts. All of the petroleum ether extract was saponified in a 1-l. round-bottomed flask using a basic solution prepared by dissolving 220 g. (3.9 moles) of potassium hydroxide in 500 ml. of 50 per cent aqueous methanol. The flask was fitted with a reflux condenser and the solution was warmed on a steam bath under reflux for 22 hr.

The basic solution was cooled, transferred to a 2-l. round-bottomed flask, diluted with 500 ml. of water, and extracted continuously with diethyl ether. Ether fractions were taken at the end of three, four, and five days. The fractions were dried overnight with magnesium sulfate and filtered with considerable difficulty. The oily filtrates were combined and concentrated to a volume of ca. 100 ml. by distillation at atmospheric pressure. The oily residue, which appeared to be emulsified with residual water, was redissolved in 300 ml. of chloroform, dried overnight with

sodium sulfate and filtered. Removal of solvent yielded a light brown glass, which, when ground with a mortar and pestle, gave 37.4962 g. of a yellow powder. When a small amount was dissolved in water (it was difficultly soluble), an opalescent solution resulted. When a small amount of the yellow powder was ignited in a porcelain spoon a white ash remained that was soluble in water and produced a basic solution (pH 11). An infrared spectrum was recorded (KBr) and showed λ_{max} 3.40, 3.46, 6.34, and 6.93 μ.

After 0.5570 g. was dissolved in five milliliters of pyridine in a 40-ml. centrifuge tube, and the yellow solution made acidic (pH 1) with 20 per cent hydrochloric acid, extraction with two 10-ml. portions of diethyl ether gave rise to an orange oil. The infrared spectrum (liquid film) showed λ_{max} 3.70, 5.84, 10.60, and 11.40 μ, among others.

That the powdered extract was moderately soluble in ether, and was not merely the result of emulsification was shown by the extraction of 10.0 g. of it in a Soxhlet extractor overnight with diethyl ether; all but 0.67 g. was extracted. The ether was removed from the solution and the glassy residue was dissolved in 10 per cent aqueous sodium hydroxide. The basic solution was then acidified to pH 1 with concentrated hydrochloric acid, extracted twice with 50-ml. portions of ether, and the extract was dried with sodium sulfate. A yellow oil (6.1948 g.) was produced after removal of solvent; the n.m.r. spectrum (10%, deuteriochloroform) showed absorptions at 1.28, 4.63 (triplet, J = 5) 6.96-8.20, 8.73, and 9.12 τ. The n.m.r. spectrum was again recorded after the addition of three drops of deuterium oxide to the sample tube; the absence of an absorption at 1.28 τ and the presence of a weak absorption at 5.33 τ were noted.

Chloroform Extracts. The oil that resulted from chloroform extraction of the fruit kernels was saponified as before using 15 g. (2.67 moles) of potassium hydroxide dissolved in 200 ml. of 50 per cent aqueous methanol. The saponified mixture was processed in a manner similar to that described for the petroleum ether extracts. This treatment yielded 3.3573 g. of "nonsaponifiable" material which was an orange colored oil. An infrared spectrum was recorded (liquid film) and showed λ_{max} 3.40, 3.48, 5.73, 6.85, 7.31, and 8.59 μ. An n.m.r. spectrum (10%, deuteriochloroform) revealed absorptions at 5.07, 8.32, and 8.84 τ. It was not examined further.

Fatty Acids

The fatty acids from the petroleum ether and chloroform extracts of the fruit kernels were isolated and esterified according to procedures previously mentioned (cf. p. 31). A 38.0 mg. sample of fatty acids derived from petroleum ether extractives gave, on esterification, 36.1 mg. of methyl esters. Likewise, 30.5 mg. of fatty acids from chloroform extractives yielded 16.9 mg. of methyl esters; the esters were obtained as colorless oils, whereas the fatty acid mixtures were yellow oils. Only ca. half of the product of esterification of the fatty acids derived from the chloroform extractives was found to be soluble in petroleum ether. The balance of the reaction product was recovered from the bottom phase, but it has not been pursued. The two samples of methyl esters were subjected to GLC analysis. These data are contained in Tables 6 and 7.

Table 6.[a] GLC of Methyl Esters from Fruit Kernels-Petroleum Ether Extract.

Methyl Ester		Retention Time (t_R) EGSS-X[b]	EGS[c]	% Total Area
C_{14}	Myristate	3.4	4.3	0.3
	Unknown	4.6	6.1	4.3
C_{16}	Palmitate	6.2	7.4	24.5
C_{16}	(-2H) Palmitoleate	7.6	9.1	10.0
	Unknown	8.6	11.9	28.5
C_{18}	Stearate	11.5	12.7	7.0
C_{18}	(-2H) Oleate	13.5	15.1	25.5

a. Retention times (t_R) are relative to n-hexane used as solvent.

b. 11% EGSS-X on 100/120 mesh Chromosorb W; $t = 162^{\circ}$, p_i = 18 psig.

c. 11% EGS on 100/120 mesh Chromosorb W; L = 12 ft.; $t = 188^{\circ}$, p_i = 25 psig.

Table 7.[a] GLC of Methyl Esters from Fruit Kernels-Chloroform Extract.

Methyl Ester		EGSS-X[b]	EGS[c]	SE-30[d]	% Total Area
C_{14}	Myristate	2.9	4.6		0.4
C_{16}	Palmitate	5.2	8.2	1.0	31.0
C_{16}	(-2H) Palmitoleate	6.4	10.1		6.1
	Unknown	8.5	11.3		2.0
	Unknown	9.2	13.2		23.9
C_{18}	Stearate	9.3	14.2		5.1
C_{18}	(-2H) Oleate	11.1	16.8		28.2
C_{18}	(-4H) Linoleate	14.3	21.8		2.4
C_{20}	Arichidate	16.8	24.8	3.5	0.9

a. All retention times are relative to n-hexane used as solvent.

b. 11% EGSS-X on Chromosorb W; t = 177°, p_i = 22 psig.

c. 11% EGS on Chromosorb W, L = 12 ft.; t = 183°, p_i = 25 psig.

d. 3% SE-30 on Chromosorb W; t = 227°, p_i = 20 psig.

Lipid Constituents of Rhus Glabra Leaves

Nonsaponifiable Lipids from Petroleum Ether Extractives - Preliminary Investigation

The green leaves used in this preliminary investigation were gathered in late May, 1963. After air-drying for a period of one week, the leaves were brittle, but not badly discolored. Stems and other extraneous matter were mechanically removed; the leaves were crushed by hand, then placed in a ball mill and ground to a fine, pale green powder. The powdered leaves were extracted by the procedures used in the fruit extractions.

A sample of the powder (2,054 g.) was continuously extracted with petroleum ether (b.p. 30-60°) for six days. The extract was first concentrated at atmospheric pressure, then evaporated to dryness *in vacuo* and yielded 101.3 g. of a dark green semisolid. The extraction was continued for an additional two days using chloroform. Removal of the solvent as before yielded 54.0 g. of extract very similar in appearance to that obtained using petroleum ether. Continued extraction with 95 per cent ethanol yielded a large quantity of extract (it was not weighed directly).

A portion (91.8 g.) of the petroleum ether extract was saponified in a 1-l. round-bottomed flask using a solution prepared by dissolving 50 g. (1.25 moles) of sodium hydroxide (Baker, reagent grade) in 400 ml. of 50 per cent aqueous methanol. The reaction was carried out for 45 hr. under reflux using a steam bath. The contents were transferred with thorough rinsing using water and petroleum ether to a 2-l. round-bottomed flask. The mixture was diluted with 750 ml. of water, extracted continuously with

petroleum ether and subsequently extracted with diethyl ether. Fractions were taken periodically. The results of the liquid-liquid extraction are summarized below. All of the fractions were dried with anhydrous magne-

Fraction No.	Solvent	No. of Days	Weight (g.)
1	Petroleum ether	3	8.8810
2	" "	1	1.0552
3	" "	1	1.6714
4	Diethyl ether	4	24.6034
5	" "	4	11.3378
6	" "	4	0.5580
7	" "	6	0.7650

sium sulfate prior to the removal of solvent. A total of 48.8718 g. of nonsaponifiable material was obtained; the first fraction was obtained as a brown oil, whereas later fractions were gummy and dark green in color. Infrared analysis of fraction No. 4 showed absorptions at λ_{max} 3.33, 3.40, 5.78, 5.85, 6.46, 6.87, 8.03, and 9.98 μ (10% in carbon tetrachloride). A n.m.r. spectrum was recorded which showed poorly resolved absorptions at 7.98, 8.32, 8.40, 8.75, 9.00, and 9.13 τ (10% in deuteriochloroform). A small portion of this fraction was dissolved in chloroform and analyzed by TLC using a fluorescent plate technique. The layer (Aluminum Oxide G) was prepared using 30 g. of the adsorbent and 60 ml. of a solution prepared by dissolving 20 mg. of fluorescein in 1.2 ml. of 0.1 N

potassium hydroxide, and diluting the solution with distilled water to a volume of 60 ml. The plate was developed with 10 per cent chloroform in benzene. The R_F values were as follows: 0.00, 0.03, 0.23, 0.40, 0.54, 0.66, and 0.90. The components corresponding to R_F values of 0.00, 0.40, and 0.90 were found to fluoresce under ultraviolet light and appeared as orange spots. The other components, when visualized under ultraviolet light, appeared as violet spots on a fluorescent green background.

The infrared spectrum of fraction number one from petroleum ether extraction of the nonsaponifiable material showed λ_{max} 2.60, 3.35, 6.44, and 6.81 μ (10% in carbon tetrachloride). Fractions one-three (11.6076 g.) were dissolved in redistilled n-pentane, combined, and chromatographed on 800 g. of alumina (column dimensions: d = 3.5 cm., h = 89.5 cm.) The details of the chromatographic separation are outlined below. A total

Fraction No.	Eluting Solvent	Volume (l.)	Weight (g.)	Description
1-4	n-Pentane	2.00	0.3235	Wh. gummy solids
5-7	25% C_6H_6 in n-pentane	1.15	0.3512	Red to orange waxes
8-13	$CHCl_3$	3.00	6.1572	Orange waxes
14-16	$CHCl_3$	1.50	2.0773	Red syrups
17	Et_2O	0.50	0.0912	Red syrup
18	25% CH_3OH in Et_2O	0.50	0.1347	Black tar
19	CH_3OH		0.5351	" "

of 9.6702 g. (83%) was recovered from the column.

Fractions 10-13 were analyzed by TLC (Aluminum Oxide G impregnated with sodium fluorescein). After development of the plate with 10 per cent chloroform in benzene and visualization of the spots in ultraviolet light, the following R_F values were noted: 0.00, 0.42, and 0.92. The components of the mixture appeared as violet spots on a fluorescent green background. Although the same components appeared to be present in all of the above fractions (no. 10-13), only fractions 10 and 11 were pursued initially. These were combined (2.1276 g.) in warm chloroform; a white solid precipitated on cooling the solution. The material was recrystallized from chloroform several times, followed by crystallization from redistilled n-hexane. The white, crystalline solid (0.1941 g.) was dried for 1.5 hr. in vacuo and showed m.p. 70.7-71.5°. This material was recrystallized from n-hexane with some improvement in melting point (20.7 mg., m.p. 71.4-71.9°). An infrared spectrum of the second crop of the material (IV) (117.8 mg.) was determined and showed absorptions at λ_{max} 2.60, 2.88, 3,35, 6.83, 7.28, and 9.54 μ (20% in carbon tetrachloride). A n.m.r. spectrum revealed absorptions at 6.50, 8.22, 8.77, and 9.14 τ, with the band at 8.77 τ considerably more intense than any of the others. GLC analysis of IV in carbon tetrachloride (2.8% SE-30 on Chromosorb W; t = 228°, p_i = 20 psig.) showed a major component at t_R = 3.0 and two impurities (t_R = 2.0, and 4.8). The impurity of lower retention time was found to be present in only trace amount, whereas the other represented 2.4 per cent of the total area.

Identification of IV (1-Docosanol). An additional 1.3304 g. of IV was obtained from fractions 12 and 13 (cf. alumina chromatography, p. 47). by repeated crystallization from n-hexane. A portion of this (24.8 mg.)

was sublimed at 75° and 280-320 μ. This treatment yielded 12.9 mg. of IV as a white, crystalline solid which was used in the preparation of a phenylurethane derivative. The material which had not sublimed after 23 hr. was an orange oil at room temperature and was discarded. A small amount of IV (12.9 mg.) was placed in a 15-ml. centrifuge tube with 0.06 ml. of phenyl isocyanate and warmed on a steam bath for six minutes. Excess phenyl isocyanate was removed by passing a stream of nitrogen over the mouth of the centrifuge tube with gentle warming. The white crystalline solid that remained was dissolved in warm petroleum ether (b.p. 30-60°) and centrifuged; the clear supernatant solution was pipetted into a four-milliliter test tube and cooled in an ice bath. The crystalline phenylurethane (V) (11.6 mg., m.p. 86.2-87.2°) was collected and dried in vacuo. On the basis of the data which had been obtained, IV was tentatively identified as 1-docosanol (lit. m.p. 69-70°; phenylurethane m.p. 85-86°) (26). The infrared spectrum of V was determined and showed absorptions at λ_{max} 2.80, 2.97, 5.70, 6.24, 6.56, 6.93, and 8.31 μ (3% in carbon tetrachloride).

In order to compare IV and V with authentic samples, 1-docosanol was synthesized by lithium aluminum hydride reduction of docosanoic acid. The procedure used was essentially that of Nystrom and Brown (27) for the reduction of fatty acids that are only slightly soluble in diethyl ether. The product, after several recrystallizations from acetone, showed m.p. 70.0-70.5°. The infrared spectra of IV and authentic 1-docosanol were recorded (both 1.7% in carbon tetrachloride) and were identical. The melting points of two samples prepared by admixing IV and authentic 1-docosanol in different proportions showed no depression. Two mixture

melting point determinations of V and the phenylurethane (m.p. 85.8-86.8°) prepared from authentic 1-docosanol showed no depression; the infrared spectrum of the authentic phenylurethane derivative was identical with that of V (both 2% in carbon tetrachloride).

Chromatography of Ether-Extractable Nonsaponifiable Material. A portion of the ether-extractable nonsaponifiable material (24.6034 g., cf. fraction No. 4, p. 47) was chromatographed on 2,398 g. of alumina (column dimensions: d = 5 cm., h = 137.5 cm.). Initially, benzene (freshly distilled) was used as the eluting solvent. The chromatographic separation is summarized below. The column retained an appreciable amount

Fraction No.	Solvent	Volume (l.)	Weight (g.)	Appearance
1	C_6H_6	1.0	0.0203	Yellow oil
2	"	1.0	0.6120	Red wax
3-4	5% $CHCl_3$ in C_6H_6	2.0	0.5453	" "
5-7	20% " " "	3.0	0.9107	" "
8-10	50% " " "	3.6	1.3941	" "
11-12	$CHCl_3$	2.6	0.6421	" "
13	"	1.0	1.5045	Yellow wax
14	"	1.0	1.6905	" "
15	10% CH_3OH in $CHCl_3$	1.0	1.0014	" "
16	1% " " "	1.0	0.5329	" "
17	1% " " "	0.5	0.2192	" "
18	1% " " "	0.5	7.6147	Red-brown syrup
19	CH_3OH	1.5	1.4778	Black tar

of material even after elution with 1.5 l. of methanol. A small portion of the material that had not been accounted for was recovered by unpacking the column in three sections and stirring the adsorbent with 16 per cent methanolic acetic acid in 1-l. beakers. The dark blue solutions (green when dilute) were filtered, combined, and concentrated *in vacuo* to a volume of *ca.* 250 ml. The resulting solution was diluted with 200 ml. of water and extracted several times with chloroform (6 x 50 ml.). The extracts were combined, dried with anhydrous magnesium sulfate, and evaporated to dryness, which yielded 0.5774 g. of black tar. The overall recovery of material was 74 per cent. TLC analysis of this material (Silica Gel G, developed with absolute ethanol) showed two spots at R_F 0.76 and 0.14 (brown colored before visualization with iodine). This material was not pursued further.

The fractions from alumina chromatography were then examined by TLC (Silica Gel G, developed with chloroform); these data are presented in Table 8. Fractions 15-17 and 19 were analyzed separately; development with two per cent anhydrous methanol in chloroform and visualization revealed only two spots for fractions 15-17 at R_F 0.78 and 0.59 (1-docosanol). Fraction 19 showed spots at R_F 0.51, 0.22, 0.09, and 0.03. The constituents of fraction 18 were best resolved by development with two per cent stock methanol in chloroform. The R_F values that were determined are as follows: 0.59 (1-docosanol), 0.50, 0.40, 0.17. It was noted that 1-docosanol, even when spotted in large amount on silical gel layers, did not adsorb iodine vapor to a large extent. Thus it was visualized as a yellow or light brown spot, whereas all of the other spots were dark brown or black. An infrared spectrum of fraction 18 was recorded and

Table 8. TLC of Nonsaponifiable Material from Petroleum Ether Leaf Extract.

Fraction No.	R_F								
2	0.87								
3	0.86	0.81	0.74						
5-7	0.85			0.64	0.58	0.50			
8-10	0.87					0.50		0.15	0.00
11-12						0.50	0.42		0.00
13						0.50	0.42	0.35	
14						0.50		0.35	
IV[a]								0.34	

a. 1-Docosanol (authentic).

showed λ_{max} 2.67, 2.97, 3.38, 5.77 (very weak), 6.84, 7.27, and 9.62 μ (2% in carbon tetrachloride).

Isolation of VI. Fraction 18 obtained by alumina chromatography was chromatographed on 750 g. of alumina. Eleven fractions were taken using chloroform (6.7 l.) as the eluting solvent. These fractions (5.7256 g., total) were reddish syrups having pleasant aromas. Three fractions (1.6473 g., total) were taken using one per cent methanol in chloroform (1.2 l.). The largest of these (fraction No. 14, 1.0726 g.) when evaporated to dryness was found to consist of a white crystalline solid suspended in a red, viscous liquid. Continued elution with two per cent methanol in chloroform (0.8 l.), followed by 100 per cent methanol (0.5 l.)

yielded an additional 0.4065 g. of material which was obtained as a black, viscous liquid. The recovery of starting material from alumina was virtually quantitative.

Fraction 14 was dissolved in ca. five milliliters of hot chloroform and the chloroform was allowed to evaporate slowly at room temperature. The residual red-brown supernatant solution was removed with a pipette leaving a white crystalline solid which was washed with six 1-ml. portions of cold carbon tetrachloride; the carbon tetrachloride solutions were combined with the chloroform solution. The solid was then dissolved in four milliliters of chloroform-carbon tetrachloride (1:1). The crystalline solid (VI) (0.1829 g.) produced by slow evaporation of solvent was collected by filtration; it showed m.p. 235.9-236.9° (sublimes). Two additional crops of crystalline VI were obtained from the combined solutions and filtrates that resulted from the above treatment; these were combined (0.2000 g., m.p. 231.8-233.3°). The infrared spectrum of VI showed λ_{max} 2.86, 3.36, 5.83, 6.11, 6.85, 7.36, 8.22, 8.62, 8.91, 9.19, 9.64, and 9.95 μ (pellet). TLC of VI showed only a single spot of R_F 0.21 (Silica Gel G; developed with 2% anhydrous methanol in chloroform).

The n.m.r. spectrum of VI (107 mg. in 0.6 ml. DMSO-d_6 and 0.4 ml. $CDCl_3$) was determined and showed poorly resolved absorptions at 4.84, 6.45, 7.47, 7.90-8.80, and 9.14 τ (the most intense peak in the spectrum). The ultraviolet spectrum of VI showed only end absorption.

Compound VI gave negative periodic acid and 2,4-dinitrophenylhydrazine tests and failed to decolorize bromine in carbon tetrachloride. The result of each test was indistinguishable from that of a blank under conditions where known compounds gave definite positive tests. A portion

of VI was recrystallized from methylene chloride for elemental analysis (m.p. 233.3-234.8°).

Anal. Found: C, 70.66; H, 9.37; N, none. Kuhn-Roth determinations of VI indicated 2.13 per cent C-methyl. VI was shown by analysis to contain 0.547 per cent active hydrogen.

Molecular weight determinations. A mass spectrum of VI was obtained, which indicated a molecular weight of 456. The most abundant ions and their relative intensities are given below.

m/e[a]	145	149	203	255	256	423	425	426	438	456	457
% of Base Peak	28	15	52	57	14	12	24	15	22	100	32

a. Several intense peaks were observed below m/e 130.

An attempt was made to verify the mass spectroscopic molecular weight using the vapor pressure osmometer. Since a different lot of absolute ethanol had to be used than had been used previously (cf. p. 18), another calibration curve was determined using benzil (m.p. 94.6-95.8) as solute. The plot of ΔR versus molality was constructed using the data obtained from nine solutions of benzil ranging from 0.0021 molal to 0.0980 molal; the curve which was obtained was very similar to that previously obtained. Three measurements were made on a solution prepared by dissolving 13.6 mg. of VI in 0.8080 g. of absolute ethanol. Of the three five-minute ΔR readings (6.15, 6.21, and 6.20), a value of 6.20 was selected which indicated a molality of 0.0326. From the above data the molecular weight of VI was calculated to be 517.

Attempts to prepare derivatives of VI. Since the infrared spectrum of VI exhibited a band of moderate intensity at 5.83 μ, an attempt was made to prepare an oxime derivative. Twenty-five milligrams of VI was reacted with 26 mg. of hydroxylamine hydrochloride in 0.1 ml. of pyridine and 0.5 ml. of absolute ethanol. The mixture was boiled under reflux for two hours, then evaporated to dryness and triturated with several milliliters of cold water. The off-white precipitate was filtered; attempts to crystallize it from ethanol-water and ligroin failed. The solution was evaporated to dryness and the amorphous solid (19.0 mg.) was chromatographed on 2.5 g. of silicic acid. Nothing was eluted with 15 ml. of chloroform. Further elution with two per cent methanol in chloroform yielded a small amount of a colorless, glassy solid, which was precipitated from methanol, and yielded after filtration only 4.6 mg. of an amorphous solid. After it was dried overnight in vacuo at 78° it showed m.p. 160.8-161.8. An infrared spectrum showed absorptions at λ_{max} 2.84, 3.38, 5.84, 6.89, 7.40, 8.26, 9.95, and 10.85 μ (pellet). Continued elution of the column with chloroform-methanol solutions did not yield any additional material.

An attempt was made to prepare an acetyl derivative of VI (102 mg., m.p. 231.8-233.3°) by reaction with three milliliters of acetic anhydride (Eastman, redistilled) in 10 ml. of pyridine (Matheson, Coleman and Bell, stored over KOH). After standing overnight, the mixture was gently warmed on a steam bath for 1.5 hr. The mixture was then cooled in an ice bath and diluted with 50 ml. of water. Since a precipitate was not obtained, enough sodium bicarbonate was added to the solution to change the pH from 6.0 to 7.5 and the resulting solution was extracted with four 20-ml.

portions of chloroform. The chloroform solutions were combined and washed in the presence of cracked ice with two 50-ml. portions of 0.2 N hydrochloric acid. The chloroform solution was dried with anhydrous sodium sulfate and evaporated to dryness in vacuo at 35°. The off-white, glassy solid, VII, (106.7 mg.) was soluble in petroleum ether and ethyl acetate at room temperature and soluble in methanol, ethanol, and acetone upon warming. A filterable solid could not be obtained either by cooling the above solutions in ice or dry ice-acetone.

Since TLC analysis of the mother liquors from which VI had been obtained indicated appreciable amounts of VI, these were combined and chromatographed on silicic acid. Fractions from the column were carefully monitored (TLC) and fractions containing VI that were eluted with one per cent methanol in chloroform were combined and dissolved in 50 per cent chloroform-carbon tetrachloride. Two crops of crystalline material were collected after partial evaporation of the solvent; the first (0.0911 g.) showed m.p. 233.8-235.8°; the second (0.0953 g.) showed m.p. 233.8-236.8°. Both crops of VI were combined with the remainder of the material that had been obtained earlier and the combined samples were twice sublimed at 220-230° and 130 μ. Crystallization of the final sublimate (0.2197 g., m.p. 233.8-236.8°) from methylene chloride-carbon tetrachloride yielded 0.1569 g. of fine, white needles (m.p. 236.3-238.3°C). An infrared spectrum showed no significant variance from that obtained earlier.

A small amount of VI (5.8 mg., m.p. 231.8-233.3°) was twice sublimed. The crystalline sublimate obtained at 230° and 130 μ showed m.p. 234.8-235.8°. This sample (ca. 5 mg.) was later analyzed by TLC. All of the crystalline preparations of VI previously mentioned showed only a

single spot on TLC. These experiments had been performed by spotting 2 λ of ca. a one per cent solution of VI on silica gel layers; visualizations with iodine vapor had routinely been carried out for a period of 10 min. since this treatment resulted in the formation of a single dark brown spot. TLC samples of VI and VII (2 λ each) were applied to a Silica Gel G layer. Development of this plate with five per cent methanol in chloroform, using a visualization time of one hour revealed impurities in both of these preparations. These data are given below (R_F values of impurities are inclosed in parentheses). The impurities appeared as well-

	R_F		
VI	0.33	(0.67)	
VII	(0.04)	0.58	(0.83)

defined spots, but were very pale yellow in color, whereas the primary components appeared as dark brown spots. These results also indicated that the product of acetylation (VII) contained no unreacted VI.

VII (106.7 mg.) was then chromatographed on 2.5 g. of silicic acid (column dimensions: d = 1.1 cm., h = 5 cm.) The column was eluted with chloroform followed by elution with two per cent methanol in chloroform. A total of 87.8 mg. (83%) was recovered. Those fractions which showed a single component at R_F 0.58 (Silica Gel H, 5% CH_3OH in $CHCl_3$) were combined. This material (59.9 mg.) was obtained as a colorless syrup. An n.m.r. spectrum was recorded and showed poorly resolved absorptions at 4.68, 5.73-6.72 (complex), 7.95, 8.15, 8.48, 8.74, and 9.00 τ.

Attempts were made to crystallize VII using a variety of solvents and solvent pairs; however, a crystalline solid could not be obtained. Furthermore, these manipulations resulted in a change in color of the syrupy material from colorless to yellow. Finally, the material was dissolved in one milliliter of methanol and water was added dropwise until the solution was slightly turbid at the boiling point of the solution. The solution was allowed to cool slowly, followed by cooling in dry ice-acetone. The white solid which resulted from this treatment was filtered and dried in vacuo at 78° for three hours. There was obtained 0.0171 g. of white powder; it was not birefringent under the microscope and showed m.p. 98-99°C. An infrared spectrum showed absorptions at 2.83, 3.36, 5.72 (shoulder at 5.81), 6.11, 6.83, 7.25, 7.36, 8.16, 8.56, 9.20, and 9.62 μ (pellet).

Isolation of VIII (β-Sitosterol). Alumina chromatography of the diethyl ether-extractable nonsaponifiable material (cf. p. 50) yielded one other sizable fraction that was eluted with chloroform. This material (fraction No. 5, 4.1645 g.) was chromatographed on 80 g. of silicic acid (column dimensions: d = 1 3/8 in., h = 6 in.). Fifty-four fractions were taken using chloroform as the eluting solvent. Examination of these fractions by TLC indicated that some fractionation of at least five components had occurred. Docosanol (IV) appeared to be a major constituent of the early fractions (No. 1-5, 1.2655 g.). Several crystallizations of the reddish-brown, waxy solid from methylene chloride yielded 86.9 mg. of white, crystalline 1-docosanol, m.p. 70.2-71.2° (shown by comparison of TLC behavior with an authentic sample).

Fractions 11-20 were combined (1.2949 g. of a tan semisolid and

crystallized from ligroin. Four crops of yellow crystals were collected. The dry crystalline solids were combined and dissolved in two milliliters of hot, redistilled carbon tetrachloride. Slow evaporation of the solvent at room temperature yielded a white, crystalline material (0.3785 g.) which, after drying overnight in vacuo at 78°, showed m.p. 133.7-137.2°. Recrystallization from methylene chloride gave VIII (0.1169 g., m.p. 136.1-137.8°) in good crystalline form and a second crop of lesser purity (0.0588 g., m.p. 128-133°). TLC analysis of VIII (Silica Gel G, developed with 1% CH_3OH in $CHCl_3$) showed a major component at R_F 0.60 and two impurities at R_F 0.43 and 0.26 (both visualized as small, pale yellow spots).

Nonsaponifiable Lipids from Chloroform Extractives - Isolation of IX (β-Sitosterol-β-D-glucoside)

The petroleum ether extraction of 2,054 g. of powdered Rhus glabra leaves has already been described (cf. p. 45). After extraction with petroleum ether the leaves were continuously extracted with chloroform for two days. Removal of chloroform in vacuo yielded 54 g. of a dark green, gummy solid. A portion of this mixture (38.2 g.) was saponified in the routine manner using 300 ml. of 50 per cent aqueous methanol solution containing 20 g. of sodium hydroxide. After 24 hr. the basic solution was cooled, diluted with 750 ml. of water, and transferred to a 2-l. round-bottomed flask. Continuous liquid-liquid extraction of this solution with petroleum ether yielded, after two weeks extraction, a total of 1.4289 g. of a red, viscous oil.

The above extraction was continued for an additional 21 days using diethyl ether as solvent. It was noted that after several days of extraction, a small amount of a yellow solid had precipitated in the flask

containing the ether extractives. At the end of five days, the extraction was interrupted and the receiving flask containing the ether-soluble nonsaponifiable material was replaced with a flask partially filled with fresh solvent. The ether solution was concentrated, cooled in ice, and filtered through a sintered glass funnel to remove the yellow precipitate. This material was observed to be somewhat crystalline under the microscope. The filtrate was concentrated, dried with anhydrous magnesium sulfate overnight, and evaporated to dryness *in vacuo*. Extraction with diethyl ether was interrupted twice more and the extracted material was processed in the same manner as described above. The results of this treatment are shown below. The pale yellow residue from filtration of

Fraction No.	No. of Days Extraction	Weight of Residue	Weight of Ether-Soluble Extract.
1	5	0.3252 g.	1.8408 g.
2	4	0.4624	0.6440
3	12	0.5073	trace

fraction number three was repeatedly washed with boiling diethyl ether, and yielded 0.5073 g. of a white powdery solid which was found to be insoluble in hot chloroform, carbon tetrachloride, acetone, *n*-hexane, water, and 50 per cent acetic acid. It was, however, moderately soluble in warm pyridine. A small amount was recrystallized from acetone-pyridine. The microcrystalline, white solid was collected by filtration, washed repeatedly with acetone and absolute ethanol, and air-dried overnight. The

crystalline material (IX) obtained showed m.p. 278-280° (with decomposition at ca. 290°).

A small amount (12.6 mg.) of the above material was sublimed in vacuo (25-95 μ) over a period of three days; during this time the temperature was slowly increased from 270° to 294°. The white sublimate (7.8 mg., m.p. 282.5-283° dec.) was mechanically removed from the condenser and upper portion of the sublimation cell and was analyzed.

Anal.	$C_{35}H_{60}O_6$	Calc'd: C, 72.87; H, 10.48
	(576.87)	Found : C, 71.81; H, 10.60

Approximately 0.5 g. of IX (m.p. 278-280°) was partially dissolved in 180 ml. of boiling 95 per cent ethanol. Pyridine (several milliliters) was then added dropwise with stirring until the solid completely dissolved. Slow cooling of the solution produced a white crystalline solid which was filtered, washed exhaustively with cold 95 per cent ethanol, and dried in vacuo at 120°. This first crop (0.2943 g.) showed m.p. 282.9-283.9°; an additional 84.9 mg. was obtained from the mother liquor. An infrared spectrum of IX was obtained which showed λ_{max} 2.89, 3.38, 5.82 (weak), 6.84, 7.33 μ and a broad absorption containing several bands between 8.48 and 10.37 μ. An n.m.r. spectrum was determined which showed several poorly resolved, weak absorptions between 4.54 and 6.44 τ, a broad absorption between 7.27 and 8.93 τ, and five bands at 8.99, 9.08, 9.17, 9.22, and 9.32 τ.

Compound IX was further characterized by preparation of an acetyl derivative. A portion of recrystallized IX (104.5 mg., m.p. 282.9-283.9°) was placed in a 10-ml. round-bottomed flask with two milliliters of acetic anhydride and three milliliters of pyridine. The flask was fitted

with a reflux condenser and the mixture was heated on a steam bath for three hours. The resulting solution was cooled to room temperature, transferred to another flask, and diluted with 10 ml. of cold water. This caused precipitation of a crystalline solid which was collected and dried. The acetyl derivative (X, 0.1292 g.) was obtained as fine, white needles, m.p. 167.7-169.2°. Recrystallization of X from absolute ethanol yielded 96.0 mg. of long, silky needles, m.p. 172.0-173.0°. The product was dried in vacuo overnight at 78°.

Anal.	$C_{43}H_{68}O_{10}$	Calc'd:	C, 69.32; H, 9.19; O, 21.47; N, 0.00
	(745.01)	Found :	C, 68.75; H, 8.58; N, 0.77
		Found*:	C, 69.32; H, 9.00; O, 21.63

Kuhn-Roth determination of X indicated 7.19 per cent or 3.57 C-methyl groups (based on a molecular weight of 745.01). Compound X was shown by analysis to contain no active hydrogen.

The infrared spectrum of X showed absorptions at λ_{max} 2.88, 3.38, 5.71 (shoulder at 5.82), 6.85, 6.98, 7.29, 7.36, 8.22, 8.67, 9.12, 9.52, and 11.10 μ (pellet). The n.m.r. spectrum of X was similar to that of IX except for the appearance of four new bands at 7.84, 7.94, 7.97, and 8.00 τ (13% in deuteriochloroform).

Elaboration of the Isolation and Identification Procedures

Initial Fractionation of Leaf Nonsaponifiable Materials. In order to perform a large scale fractionation of leaf nonsaponifiable materials, it was first necessary to procure additional quantities of petroleum ether and chloroform extracts. The results of solvent extraction of green

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* Another sample was later prepared (m.p. 171.5-172.5°).

leaves which were gathered on July 2, 1963, are presented below.

Weight of Leaves	Petroleum Ether Weight	No. of Days	%	Chloroform Weight	No. of Days	%
1,662 g.	143.2 g.	5	8.6	81.2 g.	8	4.9
1,150	94.0	6	8.2	47.1	4	4.1
1,920[a]	25.7	5	1.3	147.8	7	7.7

a. Leaves were gathered on August 1, 1963.

The petroleum ether extracts were combined (262.9 g. total) and saponified; the chloroform extracts (276.1 g. total) were treated similarly. Both of the saponified mixtures were cooled, transferred to three-liter round-bottomed flasks, and diluted with two liters of water. The two basic solutions were continuously extracted with diethyl ether for a period of about two weeks; the extractions were essentially complete at the end of this period. The ether extracts were dried with anhydrous magnesium sulfate. A total of 83.6711 g. of nonsaponifiable material was obtained from the petroleum ether extract, whereas the chloroform extract yielded 94.3210 g. of nonsaponifiable material.

The nonsaponifiable fractions just mentioned, in combination with smaller amounts of similar materials obtained earlier which had not been subjected to chromatography, afforded a total of 191.6529 g. of nonsaponifiable material for further study. A total of 97.3319 g. of this was derived from petroleum ether extracts; the balance (94.3210 g.) was obtained from chloroform extracts. The petroleum ether and chloroform nonsaponifiable materials were not combined; however, the results of TLC

(shown below) indicated that they were very nearly identical.

Nonsaponifiable Materials (Origin)	R_F [a]						
Petroleum ether extractives	0.08	0.26	0.46			0.89	0.95
Chloroform extractives	0.09	0.27	0.47	0.58	0.82	0.90	

a. Silica Gel G, developed with 3% methanol in chloroform.

Two silicic acid columns were prepared for chromatography of the above materials. Each column contained 2.27 kg. of silicic acid (column dimensions: d = 9.5 cm., h = 74.5 cm.); ca. 250-ml. fractions were taken from each column. These were first concentrated by distillation at atmospheric pressure, and then evaporated to dryness in vacuo. A total of 127 fractions were taken during the chromatography of the petroleum ether-extractable nonsaponifiable material; 149 fractions were taken from the other column. Every second or third fraction from both columns was analyzed by TLC. The TLC results provided a logical basis for combining many of the fractions which were obtained from both columns, without regard to which column a particular fraction had been obtained. The results of this procedure are shown in Table 9. Only solvent was obtained in the first seven fractions from both columns; the fractions (Roman numerals) in Table 9 are arranged in order of their elution from silicic acid (i.e. in order of decreasing R_F of the various constituents). Following elution with 30 per cent methanol in chloroform, both columns were eluted with 100 per cent methanol. In both cases, an appreciable amount of water had accumulated in these fractions owing to condensation on the tips

Table 9. Results of Silicic Acid Chromatography of Leaf Nonsaponifiable Materials.

Fraction No.	No. of Fractions[a] A	B	Total Weight (g.)	Eluting Solvent
XI	6	6	7.3911	$CHCl_3$
XII	2	2	2.4968	"
XIII	8	1	16.6621	"
XIV	3	6	43.2942	"
XV	14	-	13.9977	"
XVI	--	5	6.9205	"
XVII	--	15	11.6733	"
XVIII	8	14	5.5084	"
XIX	6	--	1.8232	"
XX	--	19	2.9613	$CHCl_3$, 1% CH_3OH in $CHCl_3$
XXI	27	--	7.7346	$CHCl_3$, 1% CH_3OH in $CHCl_3$
XXII	--	24	1.6194	2-5% CH_3OH in $CHCl_3$
XXIII	6	--	1.4162	2-5% CH_3OH in $CHCl_3$
XXIV	19	--	30.1590	8-10% CH_3OH in $CHCl_3$
XXV[b]	21	--	4.4615	30% CH_3OH in $CHCl_3$
XXVI[b]	--	29	17.3445	5-30% CH_3OH in $CHCl_3$
XXVII	--	11	0.3396	30% CH_3OH in $CHCl_3$
XXVIII[b]	--	10	1.3312	30% CH_3OH in $CHCl_3$, CH_3OH

a. A and B represent silicic acid columns containing non-saponifiable materials obtained by extraction with petroleum ether and chloroform, respectively.

b. These fractions were not combined.

of the columns. In the case of A (cf. Table 8), ca. half of the methanol eluent was discarded and the recovery of starting material from this column was found to be 88 per cent. In the case of B, all of the methanol eluents were collected and evaporated to dryness; the total recovery of starting material from this column was 98 per cent.

The results of TLC analyses of some of these fractions are given in Table 10.

Table 10. TLC of Chromatographed Leaf Nonsaponifiable Materials.

Fraction No.	R_F Values[a]	Developing Solvent
XI	0.04, 0.28 (orange), 0.90	2% C_6H_6 in n-hexane
XII	0.49, 0.60, 0.74 (orange)	15% Et_2O in n-hexane
XIII	0.16, 0.24, 0.30, 0.43, 0.60, 0.65	15% Et_2O in n-hexane
XIV	0.16, 0.24, 0.30	15% Et_2O in n-hexane
XV	0.31, 0.39, 0.50, 0.79, 0.82	30% Et_2O in n-hexane
XVI	0.16, 0.24, 0.30, 0.33	15% Et_2O in n-hexane
XVII	0.31, 0.39, 0.50, 0.79, 0.82	30% Et_2O in n-hexane
XVIII	0.24, 0.31, 0.39, 0.50, 0.79, 0.82	30% Et_2O in n-hexane
XIX	0.63, 0.76, 0.83	50% Et_2O in n-pentane
XX	0.63, 0.76, 0.83	50% Et_2O in n-pentane
XXI	0.63, 0.76, 0.83	50% Et_2O in n-pentane
XXII	0.55, 0.78, 0.87	2% CH_3OH in $CHCl_3$
XXIII	0.00, 0.79	50% Et_2O in n-pentane
XXIV	0.16, 0.32, 0.57, 0.70	5% CH_3OH in $CHCl_3$

a. The colors indicated refer to the color of the spot before visualization with iodine vapor.

Fatty Alcohols. All of the above fractions were stored as concentrated chloroform solutions. It was noticed that as the chloroform slowly evaporated from the corked flasks containing fractions XV-XVIII, a large amount of a white solid began to crystallize. This material was thought to be 1-docosanol since all of these particular fractions showed a major component by TLC of R_F 0.31 (1-docosanol) (cf. Table 9). Fraction XV was later analyzed by GLC for the purpose of identifying any other fatty alcohols which might be present. To accomplish this, several fatty alcohols were purchased to serve as standards; these compounds were 1-dodecanol, 1-hexacosanol, 1-octadecanol, 1-eicosanol, and 1-hexacosanol. Samples procured from Applied Science Laboratories were used as received; fatty alcohols purchased from other suppliers were recrystallized several times from redistilled acetone. 1-Docosanol and 1-tetracosanol were both prepared by lithium aluminum hydride reduction of the corresponding fatty acids (27).

The chloroform mixture (XV) containing the leaf fatty alcohols was first warmed on a steam bath until a solution resulted. A small aliquot was removed, evaporated to dryness, and dissolved in redistilled n-hexane for GLC analysis. GLC was performed on a three per cent SE-30 column ($t = 218^{\circ}$, $p_i = 15$ psig). The retention times (t_R) which were observed were compared with those of a mixture containing C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, C_{24}, and C_{26} straight chain, primary alcohols. The data are shown in Table 11.

Isolation and Identification of VIII (β-Sitosterol). Fractions XIX-XXII were reexamined by TLC (Silica Gel G, developed with 2% CH_3OH in $CHCl_3$). As was noted earlier (cf. Table 9), the same three constituents

Table 11. GLC Analysis of Leaf Fatty Alcohols.

Fatty Alcohol	Retention Time (t_R)		% Total
	Standard Mixture	XV	Area (XV
C_{18} 1-Octadecanol	2.3		
Unknown		2.6	15.2
C_{20} 1-Eicosanol	4.2	4.2	5.7
C_{22} 1-Docosanol	7.6	7.6	72.4
C_{24} 1-Tetracosanol	13.8	13.9	6.7

appeared to be present in each fraction; the R_F values that were observed were 0.55, 0.78, and 0.87. Compound VIII (m.p. 136.1-137.8°), which had been isolated in the course of a preliminary investigation (cf. p. 58), also exhibited an R_F value of 0.55 under these same conditions. Use of the above solvent system revealed two impurities in this preparation of VIII having R_F values of 0.20 and 0.33. Fractions XIX-XXII were each partially dissolved in boiling methanol. Enough chloroform was added to each sample to effect its complete solution. When each of the above solutions were cooled to room temperature, it was noted that a small amount of solid crystallized from solution. The crystalline material from each fraction, after filtration, was found to be contaminated with a brown, gummy residue. In each case, the crystalline material was redissolved on the funnel with hot chloroform-methanol. Repetition of this procedure several times, and cooling of the resulting solutions in a refrigerator, yielded crystalline solids of varying purity. These crystalline residues

were filterable, so the melting point behavior of each sample was examined. These results are shown below. The residue derived from fraction

Fraction No.	Weight of Residue	m.p.
XIX	0.6006 g.	amorphous
XX	1.3149	132-138°
XXI	2.5040	60°
XXII	0.2453	124.3-135°

XIX, and all of the filtrates that resulted from the above treatment were combined in acetone. Slow evaporation of the acetone yielded 104 mg. of material that melted at ca. 115°; treatment of this material with charcoal in absolute ethanol solution resulted in considerable purification (96.6 mg., m.p. 129.3-135°). The residue obtained from fraction XX was decolorized and recrystallized from absolute ethanol; this yielded 1.0231 g. of white crystalline VIII, m.p. 136.5-138.5°. This procedure when applied to XXII gave an additional 297 mg. of VIII, m.p. 129.3-137.0°.

All of the filtrates that resulted from the above operations and XXI were chromatographed on 200 g. of silicic acid (column dimensions: d = 4.5 cm., h = 22.2 cm.). A total of 33 75-ml. fractions were collected by eluting the column with chloroform and chloroform-methanol mixtures; the process of combining fractions was monitored by TLC. Fractions 11-18 (1.7967 g.), which had been eluted with chloroform, were combined and crystallized from absolute ethanol. Recrystallization of this material from ethanol gave 0.8215 g. of crystalline VIII which showed m.p. 136.5-138.4°. Again, the filtrates that resulted were chromatographed

on 15 g. of silicic acid (column dimensions: d = 2.2 cm., h = 14.2 cm.). There was obtained after chromatography and recrystallization 0.6890 g. of VIII, m.p. 134-137°, and a second crop (0.1522 g.) that showed m.p. 130-134°.

A portion of VIII (423.6 mg., m.p. 136.5-138.5°) was twice recrystallized from 95 per cent ethanol. This yielded 199.0 mg., m.p. 137.2-138.5°, $[\alpha]_D^{27}$ -33.2° (c 2.0, chloroform) [lit. (28) m.p. 139-140°, $[\alpha]_D$ -33°].

Anal.	$C_{29}H_{50}O$	Calc'd:	C, 83.99; H, 12.15
	(414.72)	Found :	C, 84.26; H, 11.67

An infrared spectrum of VIII showed absorptions at 2.84, 3.35, 5.82 (weak), 6.83, 7.34, and 9.45 μ (pellet). The n.m.r. spectrum of VIII was recorded and showed poorly resolved bands at 4.67, 6.52 (broad), 7.58-8.93 (complex), 9.00, 9.12, 9.23, and 9.33 τ. The spectrum was integrated; by assigning a value of one hydrogen to the signal at lowest field, it was determined that VIII possessed ca. 55 hydrogen atoms. The mass spectrum of VIII was determined. The spectrum indicated the presence of the molecular ion at an m/e value of 414. Compound VIII was found to decolorize bromine in carbon tetrachloride and gave a positive Bayer's test. It failed to react with 2,4-dinitrophenylhydrazine.

A small portion of VIII (108.7 mg., m.p. 136.5-138.4°) was acetylated by boiling a solution of it with 0.4 ml. of acetic anhydride and one milliliter of pyridine. After 1.5 hr. the solution was cooled, diluted with 10 ml. of water, and filtered to remove a white precipitate. The filtrate was extracted several times with 10-ml. portions of methylene chloride. The extracts were then combined with the residue and the

resulting solution was washed with 2 N hydrochloric acid, sodium bicarbonate solution, and water. The solution was dried with anhydrous sodium sulfate, evaporated to dryness, and gave 123.3 mg. of an off-white, crystalline solid, m.p. 121.8-124.7°. After one recrystallization from methanol, the acetyl derivative (74.3 mg.) exhibited m.p. 123.3-126.2°, $[\alpha]_D^{27}$ -37.7° (c 2.00, chloroform) [lit. (29) m.p. 125-126°, $[\alpha]_D$ -40°]. The infrared spectrum of the acetyl derivative showed absorptions at 3.37, 5.74, 6.83, 7.33, 8.06, and 9.71 μ (10% in carbon tetrachloride). An n.m.r. spectrum of the derivative was similar to that of VIII, except for the appearance of a sharp singlet at 8.00 τ (20% in deuteriochloroform).

TLC analysis (Silica Gel G, developed with 1% CH_3OH in $CHCl_3$) of two preparations of VIII indicated the presence of a single component at R_F 0.43. A preliminary GLC examination of these samples, however, revealed two partially resolved impurities of slightly lower retention times than that of VIII.

The trimethylsilyl ether derivative of VIII (m.p. 136.5-138.4°) was prepared (30) by reacting 2.3 mg. of VIII with 0.04 ml. of hexamethyldisilazane and 0.02 ml. of trimethylchlorosilane, in 0.2 ml. of pyridine (redistilled, stored over KOH). Compounds VIII, VIII-OAc, and VIII-$OSi(CH_3)_3$ were then analyzed using a three per cent SE-30 column under several conditions of t and p_i; the results are given below.

Further proof of the identity of VIII was obtained by comparing its GLC retention time with that of an authentic sample (Applied Science Laboratories mixture No. X-041, supplied as a mixture of β- and γ-sitosterol). The infrared and n.m.r. spectra of the β- and γ-sitosterol mixture (ca.

	Retention time (t_R)[a]		
	Impurities		Main Component
VIII	22.1	23.9	27.1
VIII-OSi$(CH_3)_3$	25.6	27.6	31.7

a. $t = 246^0$, $p_i = 14.5$ psig; t_R's are relative to pyridine used as solvent.

	Retention time (t_R)[a]		
	Impurities		Main Component
VIII	19.5	20.9	24.9
VIII-OAc	25.9	28.0	32.2

a. $t = 250^0$, $p_i = 15$ psig; t_R's are relative to n-hexane used as solvent.

80% β-) were found to be very nearly identical to those obtained for VIII. The retention times (3% SE-30) for the constituents of VIII and the authentic sample are cited below.

	Retention time (t_R)[a]		
	γ-Sitosterol	Unknown	β-Sitosterol
X-041	22.3	----	27.0
VIII	22.3	22.8	27.0

a. $t = 276^0$, $p_i = 15$ psig; t_R's are relative to n-hexane used as solvent.

The per cent purity of an analytical sample of VIII (m.p. 137.5-138.7°) was ascertained by integration of the GLC chromatogram using three different methods. The results were as follows: planimeter integration, 91.7 per cent; triangulation, 88.6 per cent; use of a disc

integrator, 89.1 per cent. The purity of another preparation of VIII (m.p. 136.5-138.4°) was found to be 93.0 per cent (assayed by planimeter integration). γ-Sitosterol was estimated to constitute four to five per cent of the total sterols in the two preparations of VIII.

Identification of IX (β-Sisosteryl-β-D-glucoside). It was noted that fraction (XXVII) (cf. Table 9), which was obtained from silicic acid chromatography of the nonsaponifiable material, contained a small amount of a chloroform-insoluble white solid. After filtration and thorough washing of the residue with boiling chloroform, 27.5 mg. of crystalline IX was obtained; the material was crystalline under a microscope and showed m.p. 282-286°. Likewise, elution of the column containing the petroleum ether-soluble nonsaponifiable material with 15 per cent methanol in chloroform yielded, after filtration, 0.7338 g. of a white solid which was thought to be IX; however, this material was not examined further.

The possibility of isolating additional quantities of IX via the acetyl derivative (X) was considered. A portion of an ethanol extract of the leaves (4.8342 g.; the leaves had previously been extracted with petroleum ether and chloroform) was dissolved in 175 ml. of pyridine. The solution was cooled in an ice bath and 100 ml. of acetic anhydride was slowly added. After standing for 10 minutes, the mixture was removed from the ice bath and allowed to stand overnight at room temperature. The mixture was then heated on a steam bath for 45 minutes, cooled, and poured into 200 ml. of water. Since a filterable solid was not produced, the resulting mixture was extracted several times with 100-ml. portions of chloroform. The chloroform extracts were combined and washed

exhaustively with 2 N hydrochloric acid and water. The chloroform solution was then dried with anhydrous sodium sulfate and evaporated to dryness. This gave 2.9778 g. of a brown tar. The substance was analyzed by TLC (Silica Gel H, developed with 1% CH_3OH in $CHCl_3$); only one spot was observed in addition to some material at R_F 0.00. The R_F value of 0.40 was in perfect agreement with that of a sample of X, which was spotted on the same plate.

On attempted GLC of X (3% SE-30; t = 300°, p_i = 20 psig.) no peaks were observed. Compound X was found to be optically active; $[\alpha]_D^{27}$ -19.3° (c, 2.07, chloroform) [lit. (29) $[\alpha]_D^{25}$ -33.7° (c, 1.30, pyridine)]. It gave a negative periodic acid test and failed to react with 2,4-dinitrophenylhydrazine. It did, however, decolorize a solution of bromine in carbon tetrachloride.

Mass spectra for IX and X were determined. These results indicated a molecular weight of 414 for IX (Fig. 1) and a molecular weight of 456 for X (Fig. 2).

The molecular weight of X was determined using the vapor pressure osmometer. In this determination, redistilled benzene was used as solvent. Six solutions of benzil in benzene were prepared which ranged from 0.0099 to 0.0601 molal in concentration. A calibration curve was determined in the same manner as described previously (cf. p. 18); this was found to be linear from ca. 0.01 to 0.05 molal. Triplicate 3.5-min. readings of ΔR were made on a single solution of X (m.p. 169.9-171.7°) prepared by dissolving 25.7 mg. of X in 1.7292 g. of benzene; the observed values of ΔR were 6.37, 6.47, and 6.47. A value of ΔR of 6.47 was selected and by using the calibration curve, the concentration of the solution

was found to be 0.0199 molal. From the above data, the molecular weight of X was calculated to be 747 (theory for $C_{43}H_{68}O_{10}$ = 745.01).

Authentic samples* of β-sitosteryl-β-D-glucoside (m.p. 291.3-296.2°) and its tetraacetate (m.p. 170.7-172°) were obtained. The infrared spectrum (pellet) of the former was found to be identical to that of IX and no significant depression of the melting point resulted after admixing this material with IX. The melting point of the authentic tetraacetate of β-sitosterol-β-D-glucoside showed no depression after admixture of this sample with X.

Search for Additional Quantities of VI. It was observed that fraction XXIV (cf. Table 9) from the initial fractionation of leaf nonsaponifiable materials on silicic acid contained a component at R_F 0.32. Since VI also was found to have this R_F value under the conditions used, XXIV was further fractionated on 450 g. of silicic acid (column dimensions: d = 6.4 cm., h = 25 cm.). A total of 28.1641 g. of material (93%) was recovered from the column in 126 fractions. Of these fractions, only fractions 70-88 (3.0514 g.) were shown by TLC to contain VI (R_F 0.38; Silica Gel G, developed with 5% CH_3OH in $CHCl_3$). These fractions also contained some β-sitosterol (R_F 0.61) and a small amount of material at R_F 0.00.

An attempt was made to isolate VI as its digitonide (31). Fractions 74-83 (1.6815 g.) from chromatography were combined and dissolved in 100 ml. of 95 per cent ethanol that contained one per cent by volume

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* The author is very grateful to Lyle J. Swift of the USDA Fruit and Vegetable Products Laboratory, Winter Haven, Florida for supplying these samples.

hydrochloric acid. To the solution in a 125-ml. Erlenmeyer flask was added 0.5937 g. of digitonin (CalBioChem, A grade). The mixture was warmed on a steam bath to aid the dissolution of digitonin and then evaporated to dryness. The brown gummy residue that resulted was washed with 100 ml. of warm benzene. The residual brown semisolid was filtered with difficulty, washed exhaustively with boiling benzene, air-dried, and gave 0.3577 g. of an amorphous, tan solid. The digitonides were dissolved in 10 ml. of hot pyridine and fifty milliliters of benzene was added to the solution. The solution was then cooled and filtered to remove a gelatinous precipitate of digitonin. The benzene-pyridine solution was washed with three 60-ml. portions of 2 N hydrochloric acid, with sodium bicarbonate solution, and finally with water. The benzene layer was dried with anhydrous sodium sulfate, evaporated to dryness, and gave XXIX, 54.4 mg. of an olive green, amorphous solid.

The benzene "solution" that resulted after precipitation of the digitonides was found to be an emulsion. This was concentrated to a volume of 30 ml. and centrifuged. Two phases resulted; the upper layer was decanted and the bottom layer was washed with two small portions of benzene. The benzene washings were combined with the upper phase and the benzene was evaporated *in vacuo*; this yielded 1.6214 g. of a brown syrup. The lower phase (pH 2) had a pungent odor and on standing overnight a white solid precipitated. The mixture was treated with seven milliliters of pyridine and diluted with benzene. Two phases resulted; the upper layer was removed and washed successively with 2 N hydrochloric acid, sodium bicarbonate solution, and water, and dried (the lower phase was discarded). Removal of benzene from the above solution yielded XXX, 31.4

mg. of a tan, gummy solid.

The brown syrup (1.6214 g.) which remained after the isolation of XXIX and XXX was again treated with digitonin (1.0420 g.). Evaporation of ethanol from this solution yielded a tan, glassy solid which, after treatment with benzene and work-up, gave 1.1411 g. of digitonides and 1.1025 g. of a benzene-soluble syrup. Treatment of the digitonides with pyridine yielded 97.1 mg. of "sterols" (XXXI), obtained as a tan, amorphous solid.

The "sterol" preparations (XXIX-XXXI) from the above treatments, as well as the residual, benzene-soluble material after treatment with digitonin were examined by TLC (Silica Gel G, developed with ca. 5% CH_3OH in $CHCl_3$). These results are cited below. Mixtures XXIX, XXX, and XXXI

	R_F						
VI	(0.19)[a]		0.44				
XXIX and XXX		0.40					
XXXI			0.44				
C_6H_6 Residue	0.19	0.40		0.51	0.65	0.72	0.83

a. Impurity.

were combined (182.9 mg. total) in 95 per cent ethanol and treated with charcoal. This treatment gave 146.2 mg. of an amorphous solid that was still brown in color; it could not be crystallized.

n-Alkanes. Fraction XI (7.3911 g., cf. Table 9) from silicic acid chromatography was obtained as a red, waxy solid. The fraction was dissolved in n-hexane and chromatographed on 850 g. of neutral alumina

(d = 3.5 cm., h = 86.3 cm.). The chromatographic separation is outlined below. A total of 5.1050 g. (69%) was recovered.

Fraction No.	Solvent	Vol. (l.)	Weight (g.)	Appearance
1-42	n-hexane	1.05	2.0164	Wh. wax
----	1-20% C_6H_6 in n-hexane	4.50	------	-------
43-72	40% " " "	3.00	0.5823	Yel. grease
73-85	C_6H_6	1.30	0.5351	Brown "
-----	1-25% $CHCl_3$ in C_6H_6	2.00	------	----------
-----	$CHCl_3$	1.06	------	----------
86-106	1% CH_3OH in $CHCl_3$	2.10	1.3807	Yel.-brn. wax
107	5% CH_3OH " "	1.00	0.3188	" " "
108-127	CH_3OH	2.00	0.2723	Yel.-wh. wax

The infrared spectrum of a center cut of the white, crystalline solid which was eluted with n-hexane (fraction No. 11, 0.8059 g.) showed absorptions at λ_{max} 3.37, 3.45, 6.83, and 7.29 μ (12% in carbon tetrachloride). The various components of this mixture were identified by GLC (3% SE-30 on 100/120 mesh Chromosorb W); these results are given in Table 12.

Nonvolatile Constituents. Fractions and XIII and XIV (cf. Table 9) were obtained as viscous, reddish-brown oils. The two fractions were combined for further fractionation. An n.m.r. spectrum of this mixture showed poorly resolved absorptions at 4.84, 7.97, 8.32, 8.38, 8.72, 9.07, and 9.17 τ (50% in carbon tetrachloride). The entire mixture (ca. 59 grams) was chromatographed on 1.000 kg. of silicic acid (column dimensions: d = 6.6 cm., h = 53.7 cm.). Elution with one liter of n-hexane had no

Table 12. GLC Analysis of Leaf n-Alkanes.

n-Alkane	Retention Time (t_R) t^a = 138°	t^b = 158°	t^a = 236°	% Total Area[c]
C_{14}	2.6			trace
C_{15}	4.2			trace
C_{16}	6.7	3.0		trace
C_{17}		4.6		trace
C_{18}		7.1		trace
C_{19}		10.7	0.9	trace
C_{20}			1.2	trace
C_{21}			1.6	1.2
C_{22}			2.1	1.6
C_{23}			2.8	0.9
C_{24}			3.6	1.5
C_{25}			4.7	10.2
C_{26}			6.1	4.0
C_{27}			8.1	51.8
C_{28}			10.5	1.9
C_{29}			13.7	12.7
C_{30}			17.7	1.5
C_{31}			23.3	9.6
C_{32}			29.9	trace
C_{33}			39.3	3.2

a. p_i = 15 psig.

b. p_i = 20 psig.

c. C_{14}-C_{19} were not considered in calculating the total area; trace = < 0.5%.

effect. The bulk of the material was obtained by eluting with 4.5 l. of 10 per cent diethyl ether in n-hexane. Continued elution with diethyl ether-n-hexane solutions resulted in a total recovery of 47.8527 g. (81%) distributed in 42 fractions each of which represented ca. 200 ml. of eluent. Several of the fractions that were eluted with 10 per cent diethyl ether in n-hexane were analyzed by TLC; these data are summarized below.

Fraction No.	Weight (g.)	R_F [a]
1	0.9865	0.00, 0.30, 0.36, 0.48, 0.57, 0.70
3	17.1288	0.00, 0.33, 0.41, 0.46, 0.50, 0.57
5	2.5954	0.00, 0.34, 0.44, 0.48, 0.53
7	1.3957	0.00, 0.34, 0.41
9	0.7642	0.00, 0.33
11	0.6705	0.00, 0.33
13	0.3958	0.00, 0.33
15	0.2301	0.00, 0.33, 0.43
17	0.1918	0.00, 0.33
19	0.1599	0.00, 0.33
21	0.1128	0.00, 0.20-0.73 (streak)
23	0.0679	0.00, 0.20-0.76 (streak)

a. Silica Gel G, developed with 20% diethyl ether in n-hexane.

The infrared spectrum of fraction three (XVIII) showed absorptions at λ_{max} 2.96, 3.39, 5.77, 6.00, 6.91, 7.29, 7.97, 9.24, 10.07, and 11.98 μ (liquid film). One gram of XVIII (an orange oil) was chromatographed on 100 g. of alumina (column dimensions: d = 1.9 cm., h = 47 cm.). The column was eluted with petroleum ether (b.p. 30-60°, 600 ml.), 10 per

cent benzene in petroleum ether (300 ml.), and 20 per cent benzene in petroleum ether (300 ml.) resulted in the recovery of 0.8553 g. (88%) of material in three fractions. TLC analysis of these indicated that very little fractionation had been achieved.

A portion of XVIII (0.8161 g.) was reacted with 1.7427 g. of 3,5-dinitrobenzoyl chloride in six milliliters of pyridine. The mixture was warmed for 0.5 hr., then dissolved in chloroform. The chloroform solution was washed successively with 2 N hydrochloric acid, sodium bicarbonate solution, and water. After evaporation of the chloroform, the residue was decolorized with charcoal in ethanol. This yielded, after work-up, 0.4141 g. of a yellow oil. TLC analysis of the product showed a spot at R_F 0.00 and a large, diffuse spot at R_F 0.52 (Silica Gel G, developed with 20% diethyl ether in n-hexane). An infrared spectrum of the product showed λ_{max} 3.44, 5.83, 6.21, 6.54, 6.98, 7.54, 7.93, and 8.73 μ (liquid film), among others.

The above preparation was repeated using 1.1032 g. of XVIII and 2.4428 g. of 3,5-dinitrobenzoyl chloride. The mixture was not heated but was allowed to stand for three days at room temperature. The product was treated as before (the charcoal treatment was omitted); there was obtained, after drying the resulting chloroform solution with anhydrous sodium sulfate, 0.9793 g. of a brown oil (XXXIII). The infrared spectrum of XXXIII was essentially identical to that of the product of the first preparation. The material could not be crystallized and was not investigated further.

An attempt was made to prepare a derivative of XVIII with phthalic anhydride. A small portion of XVIII (1.1000 g.) was reacted with 1.5472 g.

of phthalic anhydride in pyridine solution. After standing for three days at room temperature, the mixture was extracted with chloroform. Work-up of these extracts yielded 1.0096 g. of a brown oil in the neutral fraction. An infrared spectrum of this material showed absorptions at λ_{max} 3.37, 5.77, 6.21 and 6.30 (weak), 6.89, 7.27, 7.78, 8.92, and 9.35 μ (liquid film). Acidification of the reaction mixture, followed by extraction with chloroform and work-up yielded 27.9 mg. of material; an infrared spectrum showed λ_{max} 3.34 (broad), 5.78 (broad), 6.23 and 6.33 (weak), 7.76, 8.86, and 9.36 μ (liquid film). The products obtained from reaction with phthalic anhydride were not investigated further.

Several attempts were made to vacuum distill small quantities of XVIII; a significant amount of distillate was never obtained. However, XVIII showed no sign of decomposition at 300° and 200 μ pressure, although some decomposition was noted on prolonged heating at 500°.

Molecular distillation of 3.5 g. of XVIII was attempted using a modified Hickman still. The distillation was carried out for 48 hr. at less than 0.1 μ pressure and 140°. The distillate was colorless at first; at the end of 48 hr. it had become yellow. The total yield of distillate was 0.4260 g. (12%). Attempts to crystallize both the residue and distillate using a variety of solvents met with failure. The results of TLC analysis of the distillate and residue are shown below (Silica Gel G, developed with 18% diethyl ether in n-hexane).

A portion of the above residue (105 mg.) was dissolved in 0.35 ml. of n-hexane and applied to a TLC plate (Silica Gel H; 20 x 20 cm., 1 mm. thickness) by means of an electromechanical applicator. The plate was developed with 15 per cent diethyl ether in n-hexane using a traveling

	R_F						
Residue	0.38				0.84	0.89	0.95
Distillate	(0.38)[a]	0.52	0.65	0.83			
Color with I_2 Vapor	brn.	yel.	yel.	yel.	brn.	brn.	brn.

a. Pale yellow in distillate.

distance of 15 cm. Several zones were indicated by exposing a two-centimeter edge of the adsorbent to iodine vapor. Seven zones of adsorbent were removed from the plate using a suction device which transferred the adsorbent directly into Soxhlet thimbles. The material was recovered from the adsorbent by extraction for several hours with chloroform. The zones which were collected and the results of TLC analysis of these fractions are given below. The fractions were obtained as yellow-brown oils; they

Zone (cm.)	TLC Analysis[a] R_F			
XVIII	0.00[b]	0.07[b]	0.16	0.75
10.9-15.0	0.00[b]	0.07[b]	0.16	0.75
8.1-10.9	0.00			
4.7-8.1	0.00[b]		0.16	
3.4-4.7	0.00[b]	0.07[b]		
1.7-3.4	0.00[b]	0.07[b]		
0.4-1.7	0.00[b]	0.07		
-0.4-0.4	0.00[b]			

a. Silica Gel H, developed with 7.5% anhydrous diethyl ether in n-hexane.

b. The dark brown coloration after visualization with iodine vapor was retained after standing in air for 24 hr.

have not been investigated further.

Carbonyl Components. It was noted that several impure preparations of β-sitosterol (VIII) and side fractions (eluted prior to VIII) from chromatography of VIII exhibited a band at λ_{max} 5.82 μ (10% in carbon tetrachloride) in the infrared region. The results of TLC analysis of three such side fractions are given below. Both of the chromatograms were de-

Aluminum Oxide G R_F	Aluminum Oxide HF_{254} R_F
(1) 0.03[a], 0.44, 0.82	0.02, 0.60
(2) 0.03[a], 0.44, 0.84	0.02, 0.60
(3) 0.04[a], 0.13[a], 0.32, 0.47, 0.75	0.02, 0.49, 0.55[b], 0.61, 0.74
VIII	0.55[b]

a. Fluoresces red.

b. Visualized with iodine vapor.

veloped with five per cent methanol in chloroform. In the first case (Aluminum Oxide G) the components were visualized by spraying with 2,4-dinitrophenylhydrazine solution, followed by oven drying at 123° for 45 min. The components appeared as dark brown spots on a yellow background. As indicated, several of the spots were found to fluoresce under 366 mμ radiation. In the second case (Aluminum Oxide HF_{254}) the components were visualized under 253.7 mμ radiation as dark spots on a fluorescent green background. This treatment did not reveal a spot at R_F 0.55 (VIII); this component was subsequently visualized with iodine vapor.

A total of 2.4621 g. of material (XXXIV) similar to the fractions

just described was available for further examination. The TLC behavior of XXXIV was examined using a Silica Gel HF_{254} layer. Development of the chromatogram with 50 per cent benzene-chloroform, followed by visualization with 253.7 mμ radiation revealed a single spot at R_F 0.00. The material was then chromatographed on 75 g. of silicic acid (column dimensions: d = 3.2 cm; h = 16.0 cm.). The column was eluted using a linear gradient of 1 l. of benzene and 1 l. of chloroform.

Sixty 40-ml. fractions were taken; a total of 2.3841 g. (97%) of material was recovered. The first 13 fractions that were eluted contained varying amounts of a white, waxy solid. The weights and results of GLC analysis of several of these are given below. The major component in

Fraction No.	Weight	Retention Time (t_R)[a]			
2	0.1236 g.				13.1
5	0.0888	1,5			
6	0.1193	1.6	2.2	5.7 (dec.)?[b]	
7	0.1192	1.6	2.2	5.6 (dec.)?[b]	
8	0.1142		2.2	3.5 (dec.)?[b]	
9	0.1096		2.2	2.7 (dec.)?[b]	

a. 3% SE-30 on 100/120 mesh Chromosorb W; t = 220°, p_i = 15 psig; t_R's are relative to n-hexane.

b. The shapes of these peaks indicated that decomposition might have occurred.

most of these fractions was found to have a retention time (t_R) of 2.2 min. Three of the above fractions were recrystallized and their melting points were determined. These results are summarized below. Fractions

Fraction No.	Solvent	Weight	m.p.
7	Ethanol	44.6 mg.	62.2-68.8°
8	2-Butanone	58.4	71-76°
9	2-Butanone	73.0	----------

seven-nine failed to decolorize solutions of bromine in carbon tetrachloride and failed to yield precipitates with 2,4-dinitrophenylhydrazine reagent. The infrared spectra of fractions seven and eight were nearly identical and showed λ_{max} 3.40, 5.86, 6.86, 7.39, 7.74, 8.24, 13.80, and 13.98 μ (pellet). An infrared spectrum of fraction nine showed absorptions at λ_{max} 3.42, 5.78, 6.86, 7.41, 8.26, 8.55, 13.80, and 13.96 μ (pellet).

Fatty Acids. Small samples of fatty acids were derived from the saponified petroleum ether and chloroform extracts by acidification and diethyl ether extraction. From 30.5 mg. of fatty acids derived from a petroleum ether extract, there was obtained 11.9 mg. of methyl esters (2,2-dimethoxypropane method, cf. p. 31). The balance of the product of esterfication was a dark green, gummy solid that was insoluble in petroleum ether, but soluble in warm methanol. Likewise, 40.2 mg. of fatty acids derived from a chloroform extract was esterfied and gave 41.3 mg. of methyl esters. In both of the above preparations, the methyl esters were obtained as brown oils which showed absorptions at λ_{max} 5.75 μ (liquid film). Small quantities of the two samples were dissolved in n-hexane and analyzed by GLC; the results are given in Tables 13 and 14.

Table 13. GLC of Methyl Esters from Rhus Glabra Leaves-Petroleum Ether Extract.

Methyl Ester		Retention Time (t_R)[a] EGSS-X[b]	EGSS-X[c]	EGS[d]	% Total Area
C_{12}	Laurate	(2.0)		(2.4)	
	Unknown	2.1		2.5	5.9
C_{14}	Myristate	3.6		4.2	12.6
C_{16}	Palmitate	6.5		7.3	43.7
C_{16}	(-2H) Palmitoleate	8.1		9.1	4.4
	Unknown	10.9		11.8	10.4
C_{18}	Stearate	11.9	4.0	12.5	4.4
C_{18}	(-2H) Oleate	(14.1)	(4.8)	(15.0)	
	Unknown		5.1	17.3	2.2
C_{18}	(-4H) Linoleate		(6.2)	(19.3)	
	Unknown		6.8	22.0	6.7
C_{20}	Arachidate		(7.0)	(22.0)	
C_{18}	(-6H) Linolenate		(8.2)		
	Unknown		8.8		5.2
C_{22}	Behenate		(12.0)		
C_{22}	(-2H) Erucate		(14.0)		
	Unknown		14.8		4.4
C_{24}	Lignocerate		(20.6)		

a. Parentheses indicate the t_R of an authentic ester.

b. 11% EGSS-X on 100/120 mesh Chromosorb W; t = 172°, p_i = 14 psig.

c. Ibid.; t = 184°, p_i = 25 psig.

d. 11% EGS on 100/120 mesh Chromosorb W; L = 12 ft.; t = 187°, p_i = 25 psig.

Table 14. GLC of Methyl Esters from Rhus Glabra Leaves-Chloroform Extract.

Methyl Ester		Retention Time (t_R)[a] EGSS-X[b]	EGSS-X[c]	EGS[d]	SE-30[e]	% Total Area
C_{12}	Laurate	2.0		2.5		1.2
C_{14}	Myristate	3.6		4.2		9.7
C_{16}	Palmitate	6.5		7.3		28.4
C_{16}	(-2H) Palmitoleate	(8.0)		(9.0)		
	Unknown	8.4		9.2		1.2
C_{18}	Stearate	11.8	4.8	12.6		2.7
C_{18}	(-2H) Oleate	14.0	5.7	15.0		3.5
C_{18}	(-4H) Linoleate		7.2	19.2		6.3
C_{20}	Arachidate		8.2	21.7	3.4	2.4
C_{18}	(-6H) Linolenate		9.7	27.0		44.6
C_{22}	Behenate		14.2		6.3	trace

a. Parentheses indicate the t_R of an authentic ester.

b. 11% EGSS-X on 100/120 mesh Chromosorb W; t = 172°, p_i = 14 psig.

c. Ibid.; t = 190°, p_i = 20 psig.

d. 11% EGS on 100/120 mesh Chromosorb W, L = 12 ft.; t = 127°, p_i = 25 psig.

e. 3% SE-30 on 100/120 mesh Chromosorb W; t = 227°, p_i = 20 psig.

CHAPTER III

DISCUSSION OF RESULTS

Rhus Glabra Fruit Constituents

An investigation of the roots of Rhus glabra (12) and folklore concerning the plant indicated that the plant might yield some physiologically active compounds having interesting chemical properties. At an early stage of the present investigation, several extracts of Rhus glabra fruit were screened for physiological activity. It was found that an acidified ethanol extract showed mild to strong antihistaminic and antianaphylaxis activity when administered to guinea pigs in large doses. An ether extract of this extract exhibited ca. one half the milligram potency observed in the case of the whole extract, but produced delayed deaths. Responses were observed in the areas of motor incoordination, muscle relaxation, and writhing.*

An ethanol extract of Rhus glabra fruit was separated into a neutral fraction and an "alkaloid" fraction. These fractions and the original ethanol extract were tested in mice. The most prominent responses were motor incoordination, peripheral vasodilation, writhing, and central hypertension. The original ethanol extract produced a greater variety of responses and was more toxic than any of the fractions prepared from it. Although Georgia farmers are known to chew sumac berries to relieve the

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* The author is very grateful to the Mead Johnson Research Center for examining these extracts for physiological activity.

pains and symptoms of rheumatism and arthritis, animal testing of Rhus glabra extracts did not reveal any worthwhile activity.

It was thought that the fruit of Rhus glabra might contain one or several alkaloids which could possibly account for the physiological activity of certain extracts. To test this hypothesis, several attempts were made (32) to isolate a basic fraction from ethanol and acidified ethanol fruit extracts. No basic material was obtained.

When an ethanol extract of the whole fruit was cooled, a crystalline compound precipitated which was found to be a potassium salt (32). On the basis of its melting point, infrared and n.m.r. spectra, and specific rotation, the compound was identified as potassium hydrogen L-malate. Further confirmation of this assignment was obtained by ion exchange of the potassium salt on Dowex-50W-X8(H^+); this treatment yielded only L-malic acid.

Several phenolic compounds were isolated in the course of the above investigation (32). An ethanol extract was acidified and extracted continuously with chloroform. After chloroform extraction, the acid-ethanol solution was made basic (pH 9.0) by the addition of sodium carbonate and extracted with chloroform and diethyl ether. Chromatography of the ether extract on alumina gave a crystalline compound. On the basis of its melting point and infrared spectrum, the compound was identified as ethyl gallate; it was further characterized by preparation of the triacetyl derivative.

After extraction of the fruit with 95 per cent ethanol, additional extracts were obtained by stirring the fruit with hot 70 per cent ethanol which had been made 0.2 N in hydrochloric acid. The acidity of the extract

was adjusted to pH 0.3 and the extract was extracted with chloroform. The chloroform extract was an oily substance and was vacuum distilled. A colorless oil was obtained (b.p. 88° at 0.85 mm.) which was identified as diethyl L-malate on the basis of its infrared and n.m.r. spectra and specific rotation (32).

Similar treatment of another acidified ethanol extract (extracted with ether vice chloroform), followed by alumina chromatography, yielded gallic acid. The compound gave a positive ferric chloride test and was identified on the basis of its melting point and infrared and n.m.r. spectra (32).

Several of the ether extracts derived from acid-ethanol fruit extracts were acetylated. Repetitive silicic acid chromatography of these acetylated materials yielded acetyl diethyl L-malate and 3-acetoxy-4,5-dihydroxybenzoic acid (32).

Although gallic acid was isolated both as the free acid and as the ethyl ester, it would appear that the formation of ethyl gallate probably resulted from the ethanolysis of gallic acid during the extraction of the fruit with acidified ethanol. For the same reason, diethyl malate may also be an artifact. The isolation of gallic and malic acids from Rhus glabra fruit is in accord with previous findings (5,6,7). It has been reported (6,7) that nearly all of the malic acid of Rhus glabra fruit is in the form of calcium acid malate, whereas malic acid was obtained in this investigation in the form of its monopotassium salt. Both of these salts have been isolated from the fruit of a very similar species, Rhus typhina (19).

Another phenol (I) was isolated during the present investigation.

This compound was extracted very slowly by continuous chloroform extraction of an ethanol extract (most of the neutral and acidic material had been removed) that was made basic by the addition of ammonium hydroxide. The compound I was conveniently obtained by vacuum sublimation of the crude chloroform extracts. The melting points of I and its triacetate were found to be 128-131° and 161-164°, respectively. The osmometric molecular weight of I was found to be 124.1. Compound I gave a positive ferric chloride test and satisfactory elemental analyses were obtained for I ($C_6H_6O_3$) and its triacetate ($C_{12}H_{12}O_6$). The identity of I was proven to be pyrogallol by comparison of the infrared spectrum of I with that of authentic material. The melting points of I and its triacetate were in agreement with those reported in the literature (33). The n.m.r. spectrum of I showed a very complex multiplet for the AB_2 system of aromatic protons. Theoretically, AB_2 spectra consist of nine lines; however, only eight lines are normally observed experimentally. For the AB_2 system of pyrogallol, $\Delta\nu$ and J have been found to be 8.6 and 8.4 cps, respectively (34). The spectrum of pyrogallol is nearly identical to a calculated spectrum for the case where $J_{AB} = \Delta\nu_{AB}$ (34).

In past years steroidal sapogenins have attracted considerable attention as precursors for sex and cortical hormones. The sapogenins are not free in nature, but occur in combined glycosidal form called saponins (35). Many plants contain little or no saponins; in others the triterpenoid saponins predominate. In 1952, a hemolytic test for the detection of steroidal saponins was devised (22). It was found that a negative hemolytic test using alcoholic plant extracts was definite proof of the absence of steroidal saponins. Since a large quantity of ethanol Rhus

glabra fruit extracts was on hand, this material was analyzed for hemolytic activity. The extract was found to give a negative test; therefore a large scale isolation of steroidal saponins was not initiated.

Since a recent determination of the lipid constituents of the fruit of Rhus glabra had not been carried out, this problem was undertaken. Extracts of the whole fruit were obtained by successive extraction with petroleum ether and chloroform. The amounts of petroleum ether extracts ranged from 3.9 to 6.4 per cent of the weights of partially dried, whole fruit; the corresponding yields of extracts obtained by chloroform extraction were 2.1-2.9 per cent.

Silicic acid chromatography of the whole chloroform extract was not fruitful; therefore the petroleum ether and chloroform extracts were saponified. A petroleum ether extract was found to contain 6.7 per cent of nonsaponifiable materials. Repetitive chromatography of most of the nonsaponifiable material revealed that ca. one-third of this material was a mixture of n-alkanes. A center cut of the crystalline mixture of n-alkanes was analyzed by GLC. The retention times for the components of a known mixture of n-alkanes were used to construct plots of carbon number versus log t_R. These plots, which were found to be linear, thus established the retention of times for several n-alkanes for which standards were not available. The naturally derived mixture was found to contain all homologues from C_{15} to C_{33} (cf. Table 1). The n-alkanes with an odd number of carbon atoms were many times more abundant than the alkanes with an even number of carbon atoms; n-nonacosane was present in largest amount.

It was noted that the mixture of isolated n-alkanes, which was

analyzed by GLC, melted over a fairly narrow range (57-59°). Hence, it is understandable that early investigators reported the isolation of a single n-alkane (14), hentriacontane ($C_{31}H_{64}$), from the fruit of Rhus glabra. The results of the present investigation have corrected this conclusion. Since only a small amount of nonsaponifiable material was obtained from the chloroform extract of the fruit, this material was not examined further.

The fatty acid constituents of both extracts were isolated. The mixture of fatty acids derived from the chloroform extractives was esterified using diazomethane. However, the method of methyl ester preparation that was routinely used was that of Radin and coworkers (24). This method, which employs 2,2-dimethoxypropane, has been reported to afford quantitative yields of fatty acid methyl esters and was convenient, nonhazardous, and free from side reactions. Both methyl ester preparations were analyzed by GLC (cf. Tables 2 and 3) and the methyl esters were identified by comparing their retention times with those of known methyl esters. Generally, when injections were made within an hour of one another and when retention times were relatively short, it was possible to reproduce the retention time of a given component within ± 0.05 min. For components having retention times greater than ca. 15 min., or when injections were made several hours apart, maximum experimental errors of ± 0.2 min. were observed. Several fatty acids, namely myristic, palmitoleic, stearic, and behenic, were identified which had not been previously reported (14). In the case of the mixture derived from the petroleum ether extractives, evidence was obtained for several unidentified fatty acids. The possibility that these were simple monounsaturated or odd

carbon number acids was ruled out. No trace of linolenic acid, previously reported by McFadden and McMurray (14), was found in these fractions.

It was desirable to compare the above results qualitatively with the analytical results of a highly purified mixture of fatty acids derived from chloroform-extractable fruit triglycerides. A small portion of the whole chloroform extract was fractionated by solvent distribution and chromatography on Unisil silicic acid. Two fractions (II and III) obtained by elution with 65 per cent benzene in petroleum ether* and benzene, respectively, were saponified and the fatty acids were converted to the methyl esters. Sterols and free fatty acids are normally eluted from Unisil silicic acid with benzene. This fact probably accounts for the larger relative amount of lignoceric acid in III as compared with II, if it is assumed that this acid occurs free in the fruit. The results of analyses of II and III are qualitatively very similar; this could be accounted for by assuming some tailing of the triglycerides during chromatography. The results of GLC analyses of these fractions are given in Table 5.

The above data contrast in several qualitative and quantitative aspects with the analytical data for fatty acids derived from total chloroform and petroleum ether fruit extracts (Tables 2 and 3). Palmitic and oleic acids together comprised 95.2 per cent of the mixture of fatty acids derived from the total chloroform extractives. The most apparent

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* Chromatography was carried out pursuant to the verbal recommendations of C. C. Sweeley, Department of Biochemistry, Graduate School of Public Health, University of Pittsburgh. Triglycerides are normally eluted with this solvent mixture.

difference is that palmitic acid comprised 90.2 and 41.8 per cent, respectively, of the total acids in the total saponifiable portions, whereas the relative abundance of this acid derived from the purified triglycerides (Table 5) ranged from 13.1 per cent (III) to 28.9 per cent (III). It appears that the preponderance of palmitic acid in the first case (Tables 2 and 3) served to mask completely the presence of linolenic acid. These results indicate that palmitic acid, in particular, must occur free in the fruit and/or esterified with large quantities of other lipid materials in addition to glycerol. On the basis of the data presented in Table 5, oleic acid appears to be the major fatty acid of the glycerol esters.

The variance in the results of quantification with regard to individual methyl esters (Tables 2,3,5) cannot be ascribed to experimental error. The planimeter that was used was found to be accurate within one part per thousand. Quantitatively prepared mixtures of methyl esters, hydrocarbons, and fatty alcohols were analyzed. Even under adverse conditions (peaks appearing in the solvent tail, components incompletely resolved), integration of the chromatograms of these mixtures resulted in maximum deviations of ca. $\pm$ 5 per cent from the theoretical mole percentages; the usual deviations were $\pm$ 2%. No attempts were made to isolate or characterize any of the unknowns that were encountered. It is possible that some of the unknowns may be hydroxy fatty acids since these have been detected in Rhus typhina extracts (19).

After prolonged extraction of the whole fruit, it was noted that the pericarps, or the waxy outer portions of the fruit, had been essentially completely extracted. Petroleum ether and chloroform extraction

of the ground kernels that remained yielded sizeable quantities of oil. These oils probably contain little or no nonsaponifiable matter. Attempted extraction of the saponified oils with ether resulted in hopeless emulsions that were stable after standing for several months. Prolonged ether extraction yielded only soap. Although efficient removal of whatever nonsaponifiable materials were present could not be accomplished, the fatty acid constituents of these mixtures were analyzed (cf. Tables 6 and 7).

Constituents of Rhus Glabra Leaves

The green Rhus glabra leaves were extracted in the same manner as described for the fruit. A small portion of an ethanol extract was tested and showed no hemolytic activity characteristic of steroidal saponins (22). As in the case of the fruit, the green leaves were found to contain no basic material (32). However, it was noted that a white, crystalline solid precipitated during the cooling of some ethanol extracts. This material was purified by recrystallization and identified on the basis of its melting point and infrared and n.m.r. spectra as myo-inositol. This assignment was confirmed by recording the n.m.r. spectrum of an authentic sample; it was found to be superimposable on that of the naturally derived material.

An attempt was made to isolate a basic fraction from the autumnal leaves. In this experiment the powdered leaves were extracted directly with ethanol, which gave 49.6 per cent of crude extract. The extract was dissolved in aqueous methanolic acetic acid and defatted by extraction with n-hexane; this treatment removed 13.4 per cent of the material. Although a gummy solid was obtained by the addition of ammonium hydroxide

to the aqueous portion, this material was insoluble in aqueous acid and was not examined further.

Since the nonsaponifiable fraction obtained from the outer portion of the fruit by petroleum ether extraction was small (6.7%) and contained over 30 per cent of n-alkanes, an investigation of the lipid constituents of the green leaves was initiated. A preliminary investigation of the petroleum ether extractives showed that 53.3 per cent of this material consisted of nonsaponifiable components. A portion of the nonsaponifiable material (23.8%) that was extractable with petroleum ether was chromatographed on alumina. A crystalline compound, IV, was obtained from chromatography which appeared to be a primary alcohol (λ_{max} 2.60, 2.68, and 9.54 μ). A phenylurethane derivative (V) was easily prepared and IV was identified as 1-docosanol by comparison of IV and V with authentic samples.

The ether-extractable portion of the nonsaponifiable material (76.2%), which was derived from the petroleum ether extractives, was also fractionated on alumina. Rechromatography of several fractions that were eluted with chloroform-methanol gave rise to two compounds, VI and VIII, in addition to 1-docosanol. Compound VIII was obtained in good crystalline form (m.p. 136.1-137.8°) after additional chromatography on silicic acid. TLC analysis of this material, however, revealed small amounts of two impurities.

Compound VI was eluted from alumina with chloroform-methanol mixtures. It could be obtained in good crystalline form only by slow evaporation of the solvent (chloroform, methylene chloride) at room temperature. Since VI appeared to contain a hydroxyl group (λ_{max} 2.86), it was

hoped that diagnostic spin-spin splitting of the hydroxyl peaks would be observed by recording the n.m.r. spectrum of VI in DMSO-d_6 (36). However, this could not be accomplished because of the limited solubility of VI in DMSO. Molecular weight determinations of VI have yielded values of 456 (from the mass spectrum) and 517 (from vapor pressure osmometer data), neither of which can be accepted with confidence due to the large discrepancy of 61 mass units.

The results of color tests indicate that VI is saturated and does not contain adjacent hydroxyl groups. Although VI exhibits a band of moderate intensity at λ_{max} 5.83, attempts to prepare an oxime and a 2,4-dinitrophenylhydrazine derivative failed. An acetyl derivative (VII) of VI was prepared, which could not be crystallized. An infrared spectrum of VII showed an ester carbonyl band at λ_{max} 5.72 μ; the original carbonyl absorption was also evident (shoulder at λ_{max} 5.81 μ). Compound VII appears to have at least one hydroxyl group that is not acetylable (λ_{max} 2.83 μ). No conclusive information was obtained from the n.m.r. spectrum concerning the number of acetylable hydroxyls. An empirical formula could not be established for VI; however, the following formulas are suggested as possibilities.

Active hydrogen analysis of VI indicated 2.5 active hydrogens. An examination of the best available preparations of VI and VII by TLC has revealed an impurity in both of these samples.

A preliminary investigation of the nonsaponifiable material that was obtained from the chloroform leaf extracts yielded another crystalline compound, IX. Compound IX was essentially insoluble in all common organic solvents except pyridine. It was found to have the empirical

Formula	% C	% H	% O	m.w.
Found	70.66	9.37	19.97	456,517
$C_{28}H_{44}O_6$	70.56	9.30	20.14	476.7
$C_{28}H_{46}O_6$	70.26	9.69	20.05	478.7
$C_{29}H_{46}O_6$	70.99	9.45	19.56	490.7
$C_{29}H_{44}O_6$	71.28	9.08	19.64	488.7
$C_{29}H_{48}O_6$	70.70	9.82	19.48	492.7
$C_{32}H_{50}O_7$	70.30	9.22	20.48	546.8
$C_{32}H_{52}O_7$	70.04	9.55	20.41	548.8

formula $C_{35}H_{60}O_6$ and readily gave an acetyl derivative (X), $C_{43}H_{68}O_{10}$. The derivative was easily characterized as a tetraacetate by the appearance of four new bands centered near 8.00 τ in the n.m.r. spectrum.

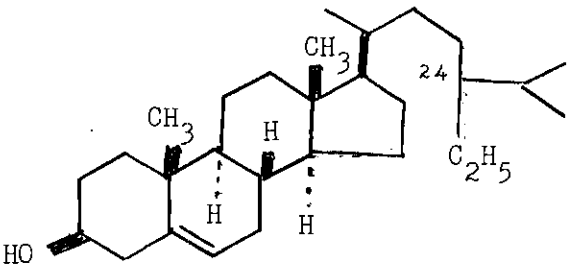
Since preliminary studies of the nonsaponifiable leaf materials had resulted in the isolation of several crystalline compounds, it was desirable to procure additional quantities of these materials for further study. The petroleum ether and chloroform extracts contained 32 per cent and 34 per cent, respectively, of nonsaponifiable material.

Alumina chromatography was not used for the initial fractionation of the nonsaponifiable lipids since in a preliminary examination this treatment resulted in appreciable irreversible adsorption of material. Instead, the nonsaponifiable materials derived from the petroleum ether and chloroform extracts were chromatographed separately on two large silicic acid columns. This treatment resulted in significant separation

of the various constituents (cf. Tables 9 and 10) and afforded good recoveries (88%, 98%) of the starting materials. TLC analyses of the fractions from the two columns provided a basis for combining many of the fractions. Fractions XV-XVIII (Table 9) consisted mainly of 1-docosanol. GLC analysis (Table 11) of XV indicated that this fraction was a mixture of 1-docosanol (72.4%), 1-eicosanol (5.7%), 1-tetracosanol (6.7%), and an unknown (15.2%). This fatty alcohol mixture constitutes ca. 20 per cent of the total nonsaponifiable lipids of the green leaves. 1-Docosanol and other fatty alcohols are common plant constituents. Recently, the C_{20}, C_{22}, and C_{24} homologues were found to be the primary wax alcohols of lodgepole pine bark; 1-docosanol was present in largest amount (31).

TLC analysis of fractions XIX-XXII showed a component having an R_F value that was identical to that of VIII. These fractions were combined and yielded, after repetitive silicic acid chromatography, more of VIII. Compound VIII ($C_{29}H_{50}O$, m.p. 136.5-138.5°), was optically active and readily decolorized bromine in carbon tetrachloride. A monoacetyl derivative of VIII (m.p. 123.3-126.2°) was easily prepared. The n.m.r. spectrum of VIII closely resembled that of cholesterol (38) and suggested that VIII was a sterol. The mass spectrum of VIII was in good agreement with that obtained earlier (39) for β-sitosterol and VIII was identified as β-sitosterol. It appears likely that β-sitosterol is identical with the sterol isolated from the fruit of Rhus glabra by McFadden and McMurray (14) in 1937.

The name sitosterin (sitosterol) was given by Burian (40) in 1897 to a sterol isolated from wheat (Greek: "sitos") and rye germs, supposed at that time to be an isomeride of cholesterol. The correct empirical



VIII. β-Sitosterol.

formula, $C_{29}H_{50}O$, was assigned by Sandqvist (41) in 1931. Several workers have contributed to the proof of identity of β-sitosterol with 22,23-dihydrostigmasterol and to the structure and configuration of the side chain (42-45). The literature concerning the sitosterols is confusing since the homogeneity of several of these sterols has been questioned. An historical account of the sitosterols and their physiological properties has been published (46). β-Sitosterol has been found to lower the blood cholestrol levels in chickens by reducing the absorption of cholesterol from the intestine into the blood stream (47). In this way, it reduces the effect of dietary cholesterol on the cholesterol content of the blood. It has been used with some success as a cardiovascular drug.

Injection of 12 μl. of hexamethyldisilazane on to the GLC column 30 min. prior to the injections of the sterols, a procedure that has been reported to reduce tailing of sterols on silicone phases (48), produced no observable effect.

The difficulty of obtaining pure β-sitosterol from plant extracts is well known. The compound readily forms mixed crystals with a variety of other sterols (46). The best preparation of β-sitosterol obtained from the leaves of Rhus glabra was 93 per cent pure, and was contaminated with

four to five per cent γ-sitosterol and a smaller amount of another sterol. γ-Sitosterol is thought to be the C_{24} epimer of β-sitosterol (44). The impurities, as well as β-sitosterol, could be converted quantitatively to the corresponding acetyl and trimethylsilyl derivatives (cf. GLC analysis, p. 72); additional evidence for the identification of VIII as β-sitosterol was obtained by comparing its GLC retention time with that of an authentic sample (cf. p. 72). Purified preparations of VIII contained no traces of stigmasterol or dihydro-β-sitosterol (sitostanol); however, various side fractions from chromatography were not analyzed for these sterols. It is interesting that pure β-sitosterol has been obtained only very recently (49).

At variance with the results of a preliminary investigation (cf. p. 59), additional quantities of IX could not be obtained directly from the diethyl ether extracts of the mixtures that resulted from saponification of crude leaf extracts. However, small quantities of IX were obtained after silicic acid chromatography and the presence of IX in ethanol leaf extracts was confirmed.

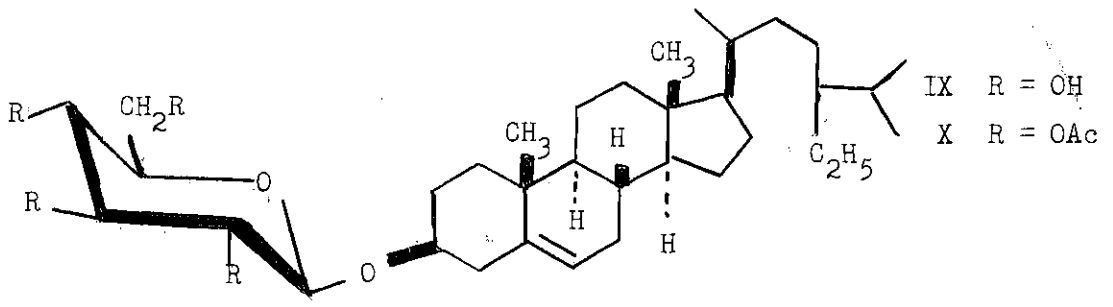
The discrepancy of 291 mass units (osmometer mw mass spectroscopic mw) of X was confusing at first. Confidence in the molecular weight (747) of X determined by use of an osmometer was obtained by determining the molecular weight of biphenyl using the same procedures and calibration curve as were used in the case of X. From the data that were obtained, a molecular weight of 153.3 was calculated (theory for $C_{12}H_{10}$ = 154.21).

This anomaly was rationalized by assuming that the peaks at m/e values of 414 and 456 in the mass spectra of IX and X, respectively, were

not due to the molecular ions. However, it was noticed that if IX consisted of a sterol, vis. β-sitosterol (m.w. 414), bonded glycosidically to a hexose, such a compound after acetylation would indeed possess a molecular weight close to 747, viz. 745. (The similarity of the mass spectra of VIII and IX were noted at a later date). A literature search resulted in the identification of IX as β-sitosteryl-β-D-glucoside. The identity of IX was confirmed by comparison of IX and X with authentic samples derived from the juice of Florida Valencia oranges (29).

β-Sitosteryl-β-D-glucoside (sitosterolin, phytosterolin, IX) is widespread in the vegetable kingdom. The D-glucoside of β-sitosterol is sometimes accompanied by the glucosides of other constituents of crude sitosterol, e.g. stigmasterol or γ-sitosterol (50). Early workers did not elucidate which anomer of D-glucose was present in intact IX. However, in one case, the sugar moiety was shown by synthesis to be β-D-glucopyranosyl (51).

The mass spectra of IX and X (Fig. 1 and 2) are worthy of some comments. Several peaks in Fig. 1 are readily assigned because of the



IX. β-Sitosteryl-β-D-glucoside.

similarity of the spectrum with that of β-sitosterol (VIII) (39). The peak of highest relative intensity in the spectrum of IX has a mass of 414 and appears to be $[C_{29}H_{50}O]^+$ (a charged β-sitosterol species). Such an ion would have to result from rearrangement of the molecular ion with migration of a hydrogen atom to the charged sterol species (52) or from collision of the species $[C_{29}H_{49}O]^+$ with the neutral molecule (IX), resulting in the abstraction of a hydrogen atom (53). A peak of low relative intensity in the spectrum of X was found to have a mass of 456. A priori, this peak might be attributed to a charged acetyl β-sitosterol species (m.w. 456). The production of this ion, if it is formed during a rearrangement process, would have to involve the migration of an acetyl or acetoxy group from some other part of the molecule. The manner in which an ion such as this could arise is not understood.

The large scale fractionation of nonsaponifiable leaf extract was expected to yield gram quantities of VI. Although the analytical and molecular weight data for VI are not compatible with a simple sterol, an attempt was made to isolate VI from crude chromatography fractions via its digitonide. The results of the first treatment of the crude material with digitonin were inconclusive since ethanol containing one per cent hydrochloric acid was inadvertently used vice ethanol. Subsequent treatment of the crude material yielded only a poorly defined precipitate that was difficult to handle. This procedure also rendered the recovered digitonin unfit for further use. The "sterol" portion that resulted from treatment with digitonin was small; however, the chromatogram (TLC) of this material did show a single component (in addition to R_F 0.0 material) of the same R_F value as VI. Compound VI was not characterized

further due to the failure in procuring additional material.

Alumina chromatography of the material first eluted (XI) from large scale silicic acid chromatography of the nonsaponifiable leaf extracts yielded a crystalline mixture of n-alkanes. The paraffinic hydrocarbon content of the total nonsaponifiable material was 1.0 per cent. GLC analysis of the mixture of n-alkanes showed that it was very similar to that derived from the fruit, although 1-heptacosane was the most abundant homologue vice 1-nonacosane (cf. Table 12).

The composition of the n-alkane mixtures derived from the fruit and leaves of Rhus glabra is remarkably similar to that of lodgepole pine bark (31), spotted bur clover (54), and other members of the vegetable kingdom. Early investigators who were concerned with elucidating the compositions of plant waxes by classical chemical methods have reported the isolation of individual paraffins (54). The range of n-alkanes and the presence of C-even alkanes have been established only very recently with the aid of modern methods (54). The results of recent studies have all indicated the predominance of n-alkanes with an odd number of carbon atoms over C-even paraffins. The reason for this is apparently unknown; however, it has been stated (31) that this phenomenon is to be expected for a biogenesis via decarboxylation of wax acids. It is of interest that the distribution of high molecular weight alkanes in Rhus glabra and other plants is quite similar to that in soils, sediments, and crude petroleum (54). Some bacteria synthesize a broad range of high molecular weight n-alkanes. However, these mixtures show no significant predominance of C-odd over C-even n-alkanes (54).

Fractions XII and XIV (cf. Table 9) together constituted the largest

portion (31%) of the total nonsaponifiable leaf material. Fraction XVIII obtained by silicic acid chromatography of this material was essentially as complex as the starting material and contained at least seven components. Since XVIII exhibited a band at λ_{max} 2.96 μ, attempts were made to prepare derivatives of the "alcoholic components;" a derivative could not be isolated. These failures probably indicate that this mixture (XVIII) is largely hydrocarbon in nature and that the absorption at 2.96 μ can be attributed to moisture. The chromatographic behavior, spectral properties, and nonvolatility of XVIII suggest that this mixture consists of high molecular weight terpenoids. The mixture has resisted fractionation by a variety of methods (adsorption chromatography, preparation of derivatives, GLC, molecular distillation, preparative TLC).

It was noted that several side fractions (higher R_F) obtained during the isolation of β-sitosterol (VIII) showed strong carbonyl absorptions in the infrared. Purification of this material by chromatography and crystallization yielded several crystalline fractions that were still mixtures. It was thought that these components might be autoxidation products of VIII, such as stigmasta-3,5-dien-7-one (55). Formation of these products from β-sitosterol parallels the behavior of cholesterol on autoxidation (56). The initial assumption concerning the ketonic nature of these components is probably not valid since the crystalline mixtures did not appear to react with 2,4-dinitrophenylhydrazine reagent; stigmasta-3,5-dien-7-one under the same conditions gave an immediate red precipitate.

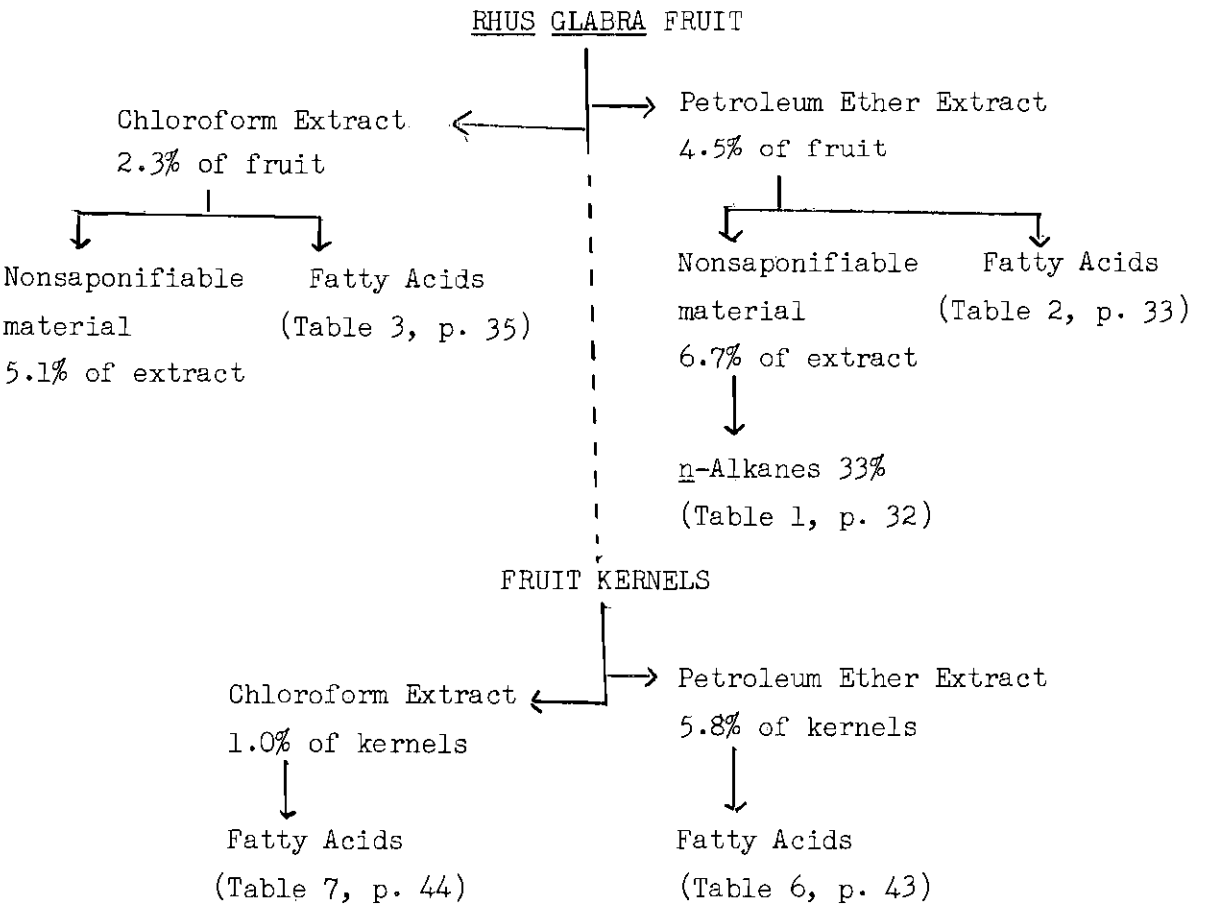
An analysis of the fatty acid constituents of the leaves has been carried out (cf. Tables 13 and 14). The methyl ester preparations were

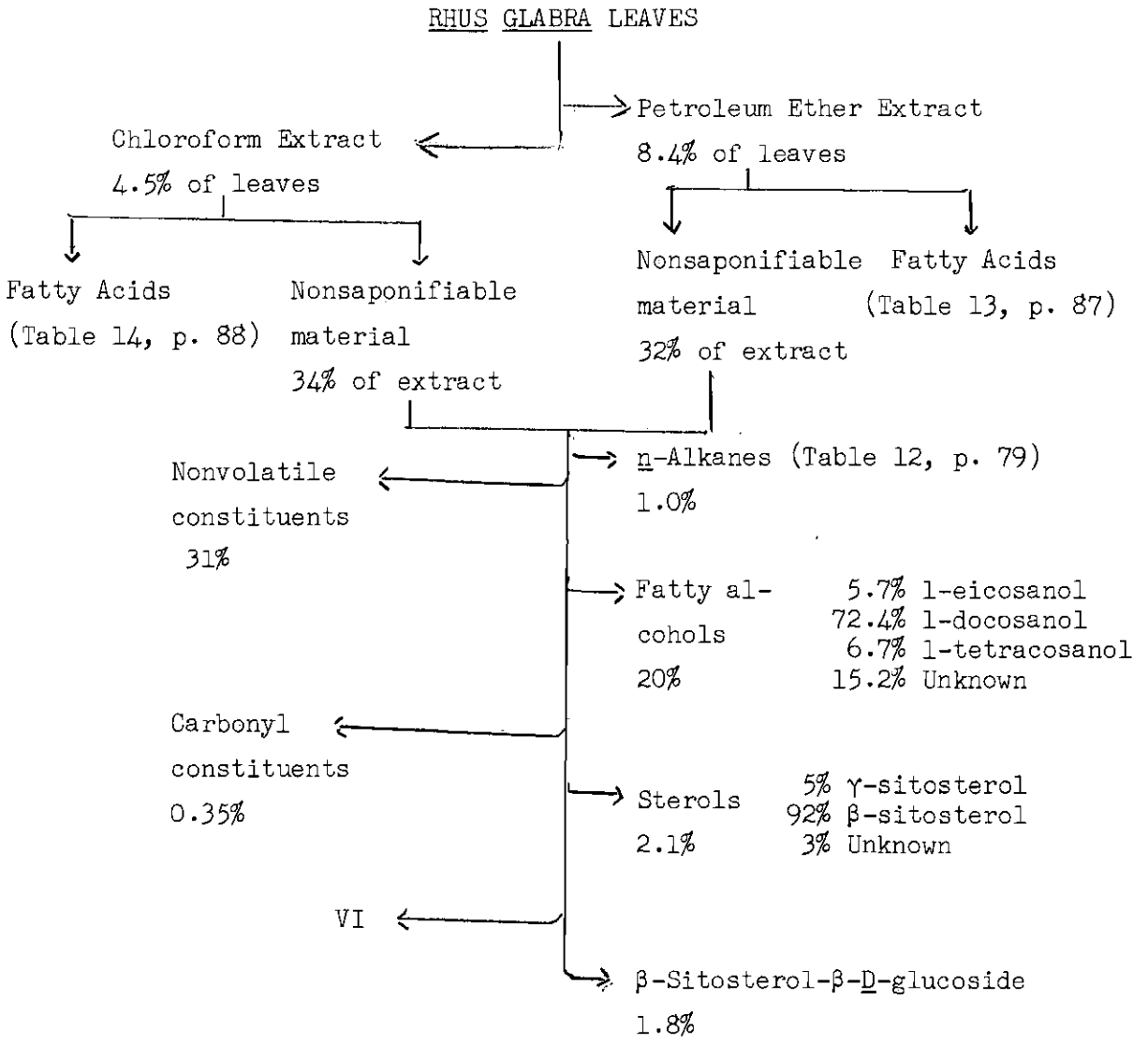
not particularly "clean" and the esterification of the acids derived from the petroleum ether extractives gave a poor yield of methyl esters. Hence, it cannot be assumed that the unknowns encountered are necessarily fatty acid methyl esters. Methyl linolenate constituted 44.6 per cent of the esters derived from the chloroform extractives. This was unexpected since only traces of linolenic acid were detected in other fatty acid fractions derived from Rhus glabra.

CHAPTER IV

CONCLUSIONS

Diagrams showing the origins and relative amounts of the constituents that were encountered in this investigation are given below.




RHUS GLABRA LEAVES
Petroleum Ether Extract
8.4% of leaves
Chloroform Extract
4.5% of leaves
Nonsaponifiable material
32% of extract
Fatty Acids (Table 13, p. 87)
Fatty Acids
(Table 14, p. 88)
Nonsaponifiable material
34% of extract
n-Alkanes (Table 12, p. 79)
1.0%
Nonvolatile constituents
31%
Fatty alcohols
20%
5.7% 1-eicosanol
72.4% 1-docosanol
6.7% 1-tetracosanol
15.2% Unknown
Carbonyl constituents
0.35%
Sterols
2.1%
5% γ-sitosterol
92% β-sitosterol
3% Unknown
VI
β-Sitosterol-β-D-glucoside
1.8%

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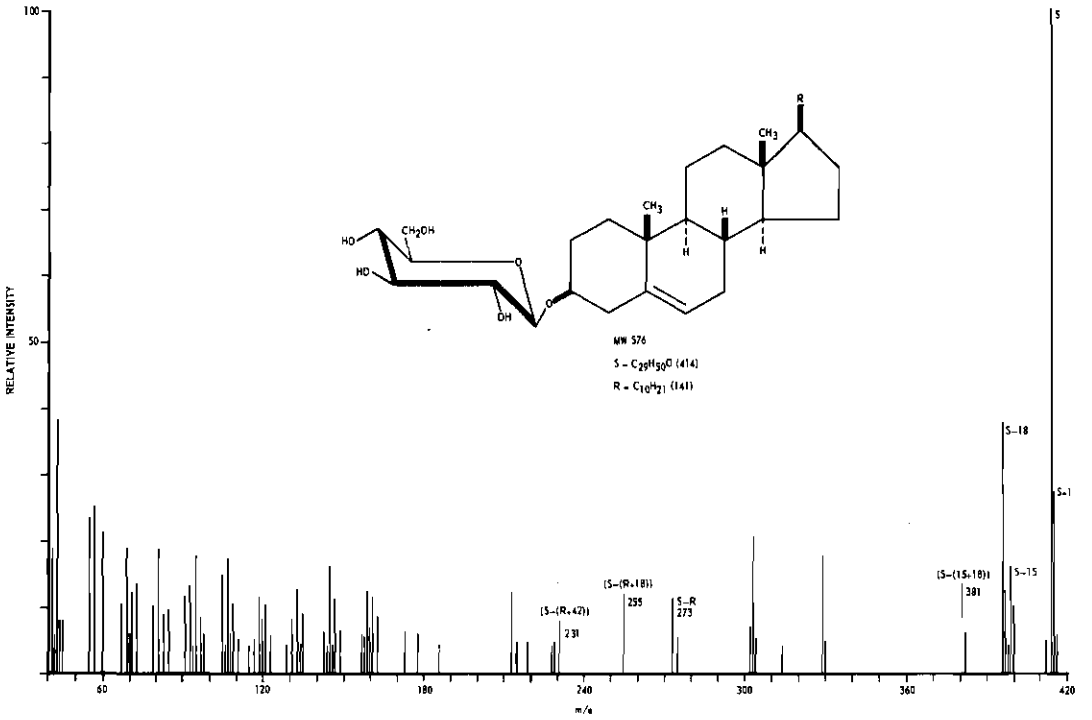

Figure 1. The Mass Spectrum of β-Sitosteryl-β-D-glucoside (IX).

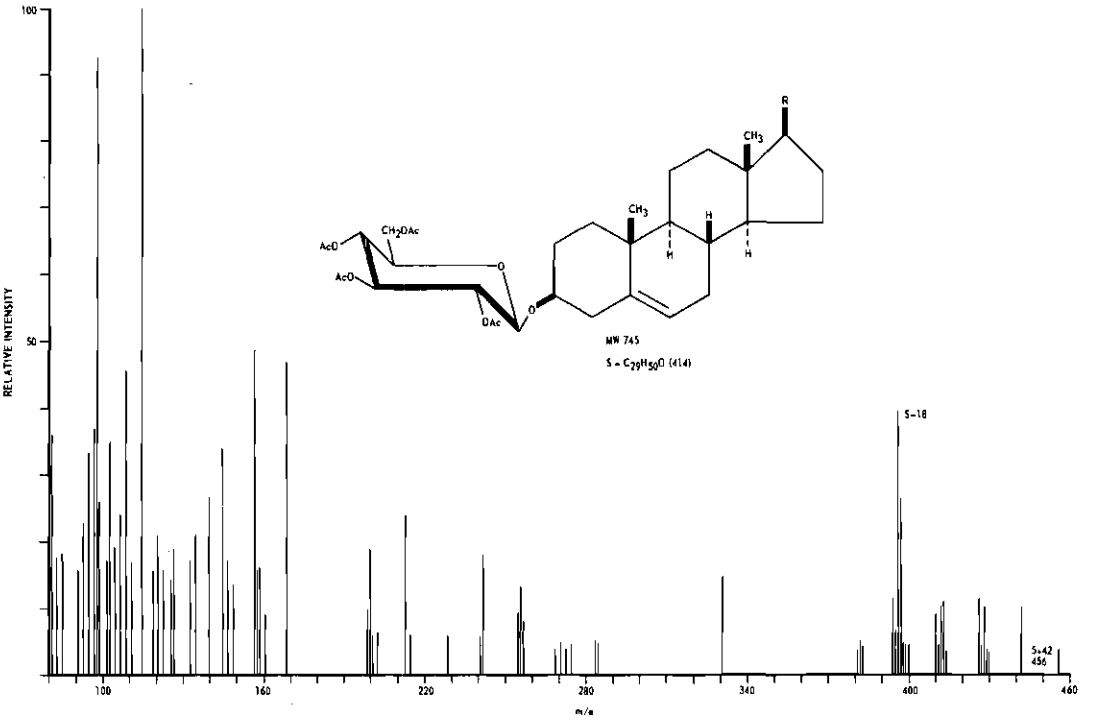

Figure 2. The Mass Spectrum of β-Sitosteryl-β-D-glucoside Tetracetate (X).

VITA

Robert Arthur Heckman was born September 4, 1937, in Dobbs Ferry, New York and attended Hastings Elementary School and Hastings High School. He entered the Georgia Institute of Technology in September, 1955, and in June, 1959 received a Bachelor of Science degree in Chemistry. The author served as a naval officer in the United States Navy from October, 1959 to September, 1961 and began graduate study at the Georgia Institute of Technology in September, 1961.

He was married on June 29, 1963 to Linda Frances Kendrick.